JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1993, p. 2513–2516
0095-1137/93/092516-04$02.00/0

Vol. 31, No. 9

Clonal Relationships among Classical *Vibrio cholerae* O1 Strains Isolated between 1961 and 1992 in Bangladesh

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Received 26 January 1993/Returned for modification 4 May 1993/Accepted 14 June 1993

In Bangladesh, the replacement of classical *Vibrio cholerae* by the El Tor biotype in 1968 and the reappearance of the classical biotype and its coexistence with the El Tor biotype after 1982 were never adequately explained. We have analyzed 23 classical *V. cholerae* isolates collected between 1961 and 1968, 14 classical isolates collected between 1982 and 1992 from the capital city, Dhaka, and 6 classical *V. cholerae* isolates collected from two southern districts of Bangladesh and studied restriction endonuclease cleavage patterns of rRNA genes (ribotypes) to investigate the clonal relationships among the isolates. Southern blots of total DNA digested with restriction enzyme *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, or *Pst*I were probed, using a cloned *Escherichia coli* rRNA operon. While restriction enzymes *Bam*HI, *Eco*RI, and *Pst*I failed to differentiate the isolates on the basis of ribotyping, *Bgl*I and *Hin*dIII produced digestion patterns that allowed differentiation. Ribotyping the isolates with *Bgl*I and *Hin*dIII revealed five different clones (ribotypes IA, IB, IIA, IC, and IIC) of classical vibrios in Bangladesh. Strains belonging to ribotypes IA and IB were isolated in Dhaka before 1968, and one ribotype (IA) was again isolated between 1982 and 1992. Ribotype IIA was isolated in 1988 and 1989, when both clones (IA and IIA) of classical vibrios coexisted with the El Tor vibrios. Isolates belonging to ribotypes IC and IIC were collected in the southern districts of Bangladesh and were clearly different from those collected in Dhaka between 1968 and 1992 by ribotyping analysis with *Bgl*I. These results support the previous assumption that classical vibrios were never completely replaced in Bangladesh and also demonstrate the existence of more than one genetically different clone of classical *V. cholerae*.

The classical biotype of *Vibrio cholerae* was presumed to be responsible for both endemic and epidemic cholera from its discovery in 1883 until about 1960. Since the present seventh cholera pandemic started in 1961, the El Tor biotype of *V. cholerae* O1 has spread extensively from Celebes (Indonesia) through Southeast Asia, southern Asia, the western coast of Africa, parts of Europe, and recently through parts of South America (5, 10, 17, 18). Although the classical biotype was responsible for the sixth pandemic, and presumably the earlier pandemics of cholera, it is thought to have been completely replaced by the El Tor biotype in all countries where the disease is prevalent, except in Bangladesh where the situation seems to be different. El Tor vibrios appeared in Bangladesh, causing the first important outbreak in 1968, and by 1973 had completely replaced the classical vibrios (1). Since then all clinical and environmental isolates in Bangladesh were of the El Tor biotype until October 1979, when five cases of cholera caused by the classical biotype were detected (8). In 1982, the classical biotype reappeared as the predominant epidemic strain in Bangladesh (13). The disappearance and reappearance of the classical vibrios in Bangladesh have never been adequately explained, and the earlier speculation that El Tor strains adapted and competed better in the environment than classical strains seems inadequate because of the reemergence of classical vibrios in Bangladesh (4, 10). Since 1982, the unusual coexistence of epidemic strains of the classical and El Tor biotypes in Bangladesh has been reported (14). These reports, however, contradicted the previous assumption that the classical biotype had completely disappeared in Bangladesh. Instead, circumstantial evidence was presented to suggest that the southern region of the country had probably always been a habitat of the classical biotype of *V. cholerae* O1 strains. To further investigate the survival of classical *V. cholerae* in Bangladesh, we used molecular techniques to determine whether the classical strains isolated between 1982 and 1992 were genetically similar to the strains collected before 1968.

A total of 43 classical *V. cholerae* isolates collected between 1961 and 1992 from cholera patients in the capital city, Dhaka, and two southern districts of Bangladesh (Bagerhat and Bhola) were included in this study. The isolates were obtained from our culture collection, and the biotypes and serotypes of all isolates used were reconfirmed by standard methods (19). All isolates were tested for antimicrobial susceptibility by the method of Bauer et al. (2) using standard antibiotic disks (BBL Microbiology Systems, Cockeysville, Md.) containing the following antibiotics and concentrations (in micrograms per disc): ampicillin, 10; chloramphenicol, 30; streptomycin, 10; tetracycline, 30; trimethoprim-sulfamethoxazole, 1.25 and 23.75, respectively; kanamycin, 30; gentamicin, 10; and nalidixic acid, 30.

Southern blot hybridization with the rRNA gene probe was performed by using the restriction enzymes *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, and *Pst*I. High-molecular-weight chromosomal DNA was isolated and digested with restriction enzymes (Bethesda Research Laboratories, Gaithersburg, Md.) as described by Stull et al. (16). The digested DNAs were electrophoresed in 0.8% agarose gels and transferred to nylon membranes (Hybond-N; Amersham International plc., Aylesbury, United Kingdom) by Southern blotting (15). The rRNA gene probe was a 7.5-kb *Bam*HI fragment of pKK3535 described previously (3, 6). The re-

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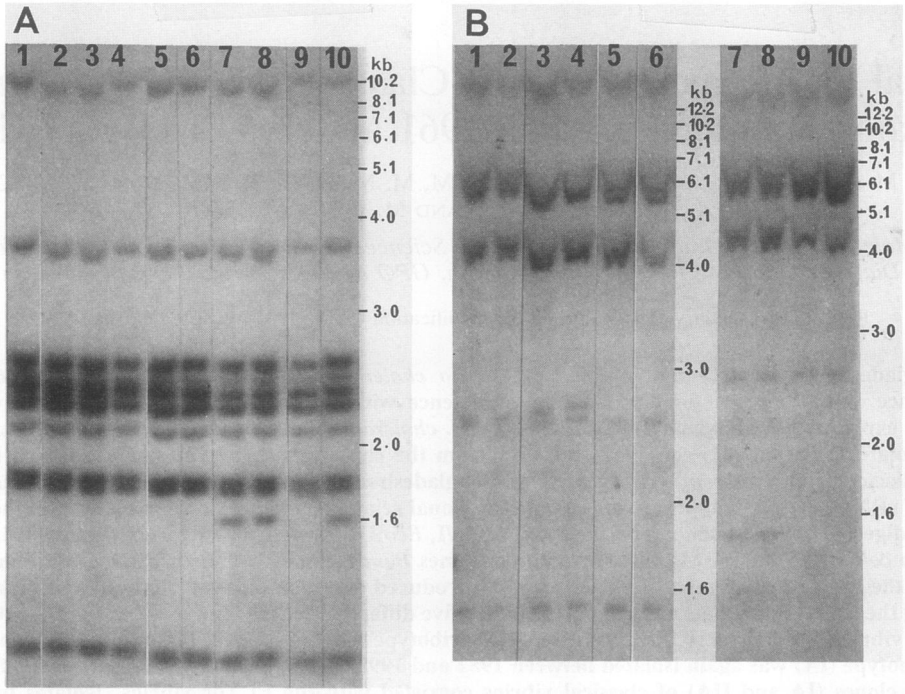

FIG. 1. Southern hybridization analysis of genomic DNA from *V. cholerae* digested with *Hin*dIII (A) and *Bgl*I (B) and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. (A) *Hin*dIII cleavage pattern of rRNA genes in four isolates collected before 1968 from Dhaka (lanes 1 through 4 [ribotype I]), four isolates collected after 1982 in Dhaka (lanes 5 and 6 [ribotype I] and lanes 7 and 8 [ribotype II]), and two isolates collected from southern districts in 1989 (lane 9 [ribotype I] and lane 10 [ribotype II]). (B) *Bgl*I cleavage pattern of rRNA genes in the same 10 isolates (lanes 1, 2 and 5 through 8 [ribotype A], lanes 3 and 4 [ribotype B], and lanes 9 and 10 [ribotype C]). Molecular sizes of bands corresponding to 1-kb DNA ladder (Bethesda Research Laboratory) are shown to the right of gels.

combinant plasmid was prepared and digested with *Bam*HI, and the insert was purified by electroelution from agarose gels as described by Maniatis et al. (11). The probe DNA was labeled by random priming (7) with [α-^{32}P]deoxycytidine triphosphate (3,000 Ci/mmol, Amersham) and a random primer DNA labeling system (Bethesda Research Laboratory). Southern blots were hybridized, washed under stringent conditions, and autoradiographed as described previously (16).

Ribotyping of the *V. cholerae* isolates produced reproducible restriction patterns, and two different ribotypes could be differentiated on the basis of the *Hin*dIII cleavage patterns of the rRNA genes of the isolates (Fig. 1A). The *Hin*dIII-generated ribotypes could be further differentiated into one or more subribotypes (A, B, and C) by using *Bgl*I (Table 1 and Fig. 1B). With each of the other three enzymes, the cleavage patterns of rRNA genes of all the isolates were similar and hence could not be differentiated (data not shown). The *Hin*dIII cleavage patterns consisted of 10 bands for ribotype I and 11 bands for ribotype II. The molecular sizes of bands ranged from 10 to 1.4 kb for both ribotypes, and ribotype II showed an additional band of 1.6 kb that was absent in ribotype I (Fig. 1A). The *Bgl*I cleavage patterns of rRNA genes consisted of seven to nine bands between 27 and 1.5 kb in size. *Hin*dIII-generated ribotype I consisted of three *Bgl*I-generated subtypes, A, B, and C, and *Hin*dIII-generated ribotype II consisted of *Bgl*I-generated subtypes A and C (Fig. 1). Considering the cleavage patterns of rRNA genes produced by cleavage with *Hin*dIII and with *Bgl*I, five genetically different clones belonging to ribotypes IA, IB, IC, IIA, and IIC were identified among the *V. cholerae* isolates (Table 1).

Of the 23 classical *V. cholerae* isolated before 1968, 15 (65.2%) belonged to ribotype IA and the remaining 8 isolates belonged to ribotype IB. All the isolates belonging to ribotype IB were collected in 1963. Of 14 isolates obtained from Dhaka between 1982 and 1992, 12 (85.7%) belonged to ribotype IA and 2 isolates belonged to ribotype IIA; of 6 isolates obtained from two southern districts, 4 belonged to ribotype IC and 2 isolates belonged to ribotype IIC (Table 1).

All of the 23 strains isolated before 1968 were susceptible to all the antibiotics tested (Table 1). Of the 20 isolates collected between 1982 and 1992, 1 isolate was resistant to streptomycin, 2 isolates were resistant to streptomycin and trimethoprim-sulfamethoxazole, and all 6 isolates collected from the southern districts were resistant to four antibiotics, tetracycline, streptomycin, trimethoprim, and sulfamethoxazole. Seven of the eight antibiotic-resistant isolates belonged to serotype Ogawa, and one isolate belonged to serotype Inaba.

All of the classical *V. cholerae* isolated before 1968 were susceptible to all the antibiotics tested, whereas a considerable proportion of the isolates collected between 1982 and 1992 were resistant to one or more antibiotics. These results showed the emergence of antibiotic-resistant classical vibrios during the 1980s and early 1990s but did not confirm whether the resistant isolates were genetically different from the endemic classical strains in terms of stable genetic markers. Similarly, previous reports that addressed the question of disappearance and reappearance of classical *V. cholerae* in Bangladesh were based on one or more phenotypic traits exhibited by isolates (9, 12), except for one study (4) which reported the persistence of a classical clone on the basis of plasmid profile analysis. However, plasmids are by

TABLE 1. Ribotypes and antibiotic resistance patterns of 43 classical *V. cholerae* isolates collected in Bangladesh from 1961 to 1992

Isolate no.	Strain	Serotype	Yr of isolation	Place of isolation	Antibiotic resistance[a]	Ribotype[b]
1	S 224	Inaba	1961	Dhaka		IA
2	S 242	Inaba	1962	Dhaka		IA
3	S 262	Inaba	1962	Dhaka		IA
4	S 263	Inaba	1962	Dhaka		IA
5	L 174	Inaba	1963	Dhaka		IB
6	L 176	Inaba	1963	Dhaka		IB
7	L 263	Inaba	1963	Dhaka		IB
8	L 333	Inaba	1963	Dhaka		IB
9	L 355	Inaba	1963	Dhaka		IB
10	L 362	Inaba	1963	Dhaka		IB
11	L 396	Inaba	1963	Dhaka		IB
12	L 547	Inaba	1963	Dhaka		IB
13	B 36921	Inaba	1964	Dhaka		IA
14	C 15375	Ogawa	1965	Dhaka		IA
15	C 15603	Ogawa	1965	Dhaka		IA
16	C 17676	Ogawa	1965	Dhaka		IA
17	C 19385	Ogawa	1965	Dhaka		IA
18	C 19751	Ogawa	1965	Dhaka		IA
19	C 19752	Ogawa	1965	Dhaka		IA
20	D 19316	Ogawa	1966	Dhaka		IA
21	E 14850	Inaba	1967	Dhaka		IA
22	E 14983	Ogawa	1967	Dhaka		IA
23	F 14372	Ogawa	1968	Dhaka		IA
24	X 20658	Inaba	1982	Dhaka		IA
25	Z 2602	Ogawa	1984	Dhaka		IA
26	Z 30979	Ogawa	1984	Dhaka		IA
27	AA 3466	Inaba	1985	Dhaka		IA
28	AA 22926	Ogawa	1985	Dhaka		IA
29	AB 8404	Inaba	1986	Dhaka		IA
30	AC 11966	Inaba	1987	Dhaka		IA
31	AC 11441	Inaba	1987	Dhaka		IA
32	AD 2700	Ogawa	1988	Dhaka		IIA
33	AD 238	Ogawa	1988	Dhaka		IIA
34	AE 4731	Ogawa	1989	Bagerhat	Tet^r SXT^r S^r	IC
35	AE 4727	Ogawa	1989	Bagerhat	Tet^r SXT^r S^r	IIC
36	AE 2883	Ogawa	1989	Bagerhat	Tet^r SXT^r S^r	IIC
37	AE 4733	Ogawa	1989	Bagerhat	Tet^r SXT^r S^r	IC
38	AE 7471	Ogawa	1989	Bhola	Tet^r SXT^r S^r	IC
39	AE 7485	Ogawa	1989	Bhola	Tet^r SXT^r S^r	IC
40	AE 3291	Ogawa	1989	Dhaka		IA
41	AG 16946	Ogawa	1991	Dhaka	SXT^r S^r	IA
42	AG 19438	Inaba	1991	Dhaka	SXT^r S^r	IA
43	AH 170	Ogawa	1992	Dhaka	S^r	IA

[a] Strains were resistant to tetracycline (Tet^r) streptomycin (S^r), and trimethoprim-sulfamethoxazole (SXT^r). All isolates were susceptible to ampicillin, chloramphenicol, gentamicin, and nalidixic acid.

[b] Ribotypes I and II were determined by using restriction enzyme *Hin*dIII, whereas ribotypes A through C were determined by using *Bgl*I. Some isolates belonging to a particular *Hin*dIII-generated ribotype could be further differentiated into more than one *Bgl*I-generated ribotype.

nature unstable genetic elements, and hence we concentrated our study on more stable genetic markers. We were able to demonstrate the presence of five distinct clones among the classical *V. cholerae* isolates on the basis of the cleavage patterns of conserved rRNA genes using restriction endonucleases *Hin*dIII and *Bgl*I. One clone (ribotype IA) appeared to be characteristic of the endemic strains isolated before 1968 and again after 1982 in Dhaka. The other clone of classical vibrios isolated before 1968 was ribotype IB, which appeared in 1963, and all eight isolates tested belonged to this ribotype. Ribotype IB differed from ribotype IA in the *Bgl*I restriction pattern of rRNA genes which showed an additional band of 2.6 kb in ribotype IB (Fig. 1). Repeated ribotyping of these isolates ruled out the possibility of any artifacts producing additional bands in the gel (Fig. 1B). Surprisingly, however, this ribotype was not detected among isolates collected in other years, and the transient appearance of this clone of classical vibrios remains to be explained. Another clone (ribotype IIA) seems to have entered this community recently and has been isolated from Dhaka

in 1988 and 1989 (Table 1). All of the six isolates from the two southern districts of Bangladesh showed identical *BglI* restriction patterns of rRNA genes which were clearly different from those of strains isolated in Dhaka between 1961 and 1992, because a 1.5-kb *BglI*-generated band which was present in type A and B was absent in type C (Fig. 1B).

The controversy over the disappearance of classical cholera in Bangladesh has given rise to two schools of thought. (i) The classical biotype of *V. cholerae* responsible for endemic cholera and perhaps the sixth cholera pandemic was completely replaced by El Tor biotype in Bangladesh and the classical strain that reappeared later on was a different clone that competed and survived better in the environment than the previous classical vibrios (9, 12, 13). (ii) The classical vibrios of the sixth pandemic were never completely replaced by the El Tor vibrios, and the classical vibrios survived in the southern region where the environment was more suitable for the classical vibrios (14). Results of the present study partially support both of these assumptions. We have demonstrated for the first time that there is indeed more than one clone of classical *V. cholerae* in Bangladesh. However, contrary to the first hypothesis, classical *V. cholerae* had not been completely replaced by the El Tor biotype and strains of the original clone (ribotype IA) have been isolated between 1982 and 1992. However, during the same period, a second clone (ribotype IIA) has also been detected in Dhaka in 1988 and 1989. Only three classical isolates between 1991 and 1992 have been analyzed in the present study and all three were of the original type (ribotype IA). Moreover, of the 14 classical *V. cholerae* isolates collected in Dhaka from 1982 to 1992, 12 isolates were of the type isolated before 1968.

Although strains isolated from Dhaka and the two southern districts appeared clonal on the basis of their *Hin*dIII ribotypes, subtyping with *BglI* revealed differences among the two groups of strains. However, Dhaka, the capital city of Bangladesh, has an enormous influx of people from both neighboring and far-off districts. Therefore, the isolation of endemic classical *V. cholerae* strains of the original clone (found before 1968) in Dhaka indicates the possible existence of the classical clone in other districts as well.

These results, therefore, are in favor of previous reports (4, 14) indicating that classical strains from the sixth pandemic were never completely replaced by the El Tor biotype and at the same time show that there is more than one clone of classical *V. cholerae* causing illness in Bangladesh. The demonstration of the transient appearance of a second clone of classical vibrio (ribotype IB) in 1963, the appearance of a new clone in 1988, the coexistence of two classical clones (ribotypes IA and IIA) with the El Tor strains, and the genetic differences among isolates from the central and southern regions of Bangladesh further complicate and deepen the mystery of the transient appearance and disappearance of classical vibrios in Bangladesh. Possible environmental factors associated with the survival and domination of different clones of classical vibrios need to be monitored in further studies.

This study was funded by the United States Agency for International Development (USAID) under grant DPE-5986-A-00-1009-00 with the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). ICDDR,B is supported by the aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, The Netherlands, Norway, Saudi Arabia, Sweden, Switzerland, United Kingdom, and the United States; international organizations including the United Nations Children's Fund, the United Nations Development Programme, the United Nations Population Fund (UNFPA) and the World Health Organization; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank Manzurul Haque for secretarial assistance.

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