J Diar Dis Res 1983 Mar;1(1):5-9
0253-8768/83$1.00+0.10

COMPARATIVE BEHAVIOUR OF CLASSICAL AND EL TOR BIOTYPES OF VIBRIO CHOLERAE O1 ISOLATED IN BANGLADESH DURING 1982

M.I. HUQ, S.C. SANYAL, A.R. SAMADI AND K.A. MONSUR

International Centre for Diarrhoeal Disease Research, Bangladesh, G P O Box 128, Dhaka 2, Bangladesh

Abstract

The change from classical to the El Tor biotype of *Vibrio cholerae* during the early seventies in Bangladesh remains to be elucidated. The shift in 1982 from El Tor back to the classical was not predicted, but provided an opportunity to study both the biotypes occurring simultaneously in nature, compared with earlier isolates. Comparative studies showed TCBS to be as good as TTGA for isolation of the two biotypes. Replacement of the El Tor by the classical biotype as the dominant epidemic strain, occurred over four months. Isolates of both biotypes from 1982 were found to be slow in mannitol fermentation. The isolates of El Tor were frankly haemolytic and belonged to phage type 4. The classical strains were of phage type 3. *V. cholerae* strains isolated in the late sixties and early seventies were similar in these markers. This suggested that the epidemic was caused by strains indigenous to Bangladesh. A classical strain of *V. cholerae* isolated in 1969 was overgrown by an El Tor strain of the same year when grown together in peptone water. Classical strains of 1982, however, grew competitively with 1969 and 1982 strains of El Tor. One classical isolate of 1982 survived for 50 days when grown with an El Tor strain of 1969. These findings suggest that the classical *V. cholerae* strains of 1982 successfully compete with the El Tor strain. They are more toxigenic than the prevailing El Tor biotype.

Key words: Vibrio cholerae; Cholera.

Introduction

The El Tor biotype of *Vibrio cholerae* was first recognized as a causative agent of cholera in Bangladesh in 1968 (2). In 1973 it replaced the classical biotype. Despite the apparent absence of the classical biotype after 1973, this Centre continued regular biotyping of *V. cholerae* strains. In 1979 (11), 1980 and 1981, several strains of classical biotype were found with El Tor remaining dominant until August 1982. A case of cholera due to classical *V. cholerae* was found on September 3, 1982. From then onwards, the isolation of this biotype increased and by the last week of December 1982, nearly 90% of the isolates were of the classical biotype.

The earlier shift from classical biotype to El Tor and the present return of classical cholera remain an enigma. The current change in biotype provided a unique opportunity to study simultaneously isolated strains of both biotypes, and compare them with strains of previous years to determine what gave the classical biotype the capacity to displace the El Tor biotype.

Materials and methods

Isolation and identification:

Freshly passed stool specimens collected in sterile glass containers, or obtained by rectal swabs placed in Cary and Blair transport medium, were transferred to the laboratory within an hour. Taurocholate tellurite gelatin (TTGA) agar (14) and Thiosulfate citrate bile salt sucrose (TCBS) agar (13) plates were inoculated by swabbing the agar surface in a circular fashion over an area of ca. 3.75 cm in diameter. After inoculating the TTGA plate, the swab was rotated to expose a fresh side prior to inoculating a TCBS plate. The plates were then streaked with a wire loop to obtain isolated colonies. MacConkey and Salmonella Shigella agar plates were inoculated similarly to exclude other enteric bacteria. All the plates were incubated overnight at 37°C.

Suspected colonies of *V. cholerae* were inoculated into several media: Kligler iron agar, motility indole urea medium, Moeller's lysine and ornithine decarboxylases and the arginine dihydrolase media, M-R V-P medium, peptone water containing 0 and 8%

sodium chloride, and Andrade's peptone water which contained 1% mannitol, sucrose, arabinose or mannose. All the tests were done following the methods of Cowan and Steel (4). The colonies were also tested for agglutination with O-group 1 and monospecific Ogawa and Inaba antisera. Biotype differentiation was done by the modified tube haemolysis method (20) and agglutination of 2.5% chicken erythrocytes (8). Sensitivity to 50 U polymyxin B (17), classical group IV (15) and El Tor Group 5 (3) phages was also tested. Phage typing of the strains of classical and El Tor biotypes was done, following the methods of Mukerjee (14) and Basu and Mukerjee (3).

Growth of the classical and El Tor biotypes of Vibrio cholerae:

To compare growth of the classical and El Tor strains isolated during the 1982 epidemic with that of the isolates obtained in earlier years, two strains of each biotype isolated in 1969 and 1982 were grown in overnight culture using T_1N_1 medium (Trypticase, 1%; sodium chloride, 0.5%; and distilled water 100 ml). The broth was diluted 10^{-4} with sterile peptone water and 1 ml amounts were added to 100 ml quantities of the same medium prepared in flasks. The inoculated flasks were incubated at 37°C. The plating of 10-fold dilutions of the cultures incubated for 0,2,4,6,8,10,12 and 24 hours was performed in duplicate on gelatin agar by spread plating and, colony forming units (cfu) were recorded following overnight incubation.

Interaction between strains of Vibrio cholerae classical and El Tor biotypes in vitro:

To determine whether the classical and El Tor strains could be grown together in the same flask culture, two strains of each biotype isolated in 1969 and 1982 were selected for study. The El Tor strain was of the Ogawa and the classical of the Inaba serovars, respectively. Overnight growth of the strains of classical Inaba and El Tor Ogawa in T_1N_1 broth were diluted serially to 10^{-6} and 1 ml of each was added to 100 ml of peptone water and incubated at 37°C. Plating for viable counts was done at specific time intervals for 24 h. The viable counts were checked by using agglutination with monospecific antisera. In one experiment with a classical biotype of 1982 and El Tor biotype of 1969, this observation for survival was continued up to 50 days.

Lysogenicity:

Thirty strains, 15 of each biotype isolated during the current epidemic, were examined for lysogenic properties. The strains were grown in T_1N_1 broth for 5 h and then treated with chloroform. The supernate was diluted serially 10-fold to 1:100 and one loopful each of the original and the diluted lysate was inoculated onto the middle of lawns of individual strains. The plates were incubated overnight at 37°C and the lawns observed for lysis or plaque formation by phage.

Toxigenicity:

Preparation of culture filtrates: Thirty-four strains, including one of each biotype, were picked each week, at random, over the 17-week study period for toxigenicity testing. Five or six colonies were picked up following overnight growth on gelatin agar and inoculated into 10 ml of Richardson's medium (16) in 50 ml conical flasks and incubated overnight in a shaking water bath (120 oscillations/min) at 37°C. The cultures were centrifuged at 4°C for 30 min at 22,000xg. The supernates were passed through membrane filters of 0.22 µm average pore diameter and stored at −20°C.

Rabbit ileal loop test; This was done following the method of De and Chatterjee (6), as modified by Annapurna and Sanyal (1) and using the above culture filtrates. Physiological saline and culture filtrates of toxigenic *V. cholerae* strain 569B prepared simultaneously, served as negative and positive controls, respectively. All experiments were performed in triplicate.

Skin permeability factor (PF): This experiment was carried out following the methods of Craig (5), Dubey and Sanyal (7) using the above culture filtrates.

Titration of toxin in culture filtrates was done for nine and six strains of each biotype isolated during 1969-72 and 1979-82, respectively. The culture filtrates were initially diluted to 1:20 and serially diluted 2-fold. To determine the titres, culture filtrates were diluted as required and tested again to measure the nearest end points (BD_4).

GM_1 ELISA and CHO cell assays: GM_1 ganglioside immunosorbent assay (20) and Chinese hamster ovarian cell assay (9) were done using 34 culture filtrates prepared from strains of the 1982 set of isolates.

Results

A total of 4401 strains of *V. cholerae* 01 were isolated from patients at the ICDDR,B Hospital, Dhaka, during September 3 to December 30, 1982 (Table 1). The growth of the organisms was readily observed on TTGA and TCBS plates. Biotyping revealed that 2620 strains were of the classical and 1781 were of the El Tor biotype. Of the classical strains, 2617 were Inaba serovar and 3 Ogawa, whereas among the El Tor, 783 were Ogawa and 998 Inaba. A steady increase in the number of weekly isolations of classical strains was noted and by the first week of December, the El Tor strains constituted less than 10% of the total isolations.

Biochemically, the isolated strains were identified as *V. cholerae* 01, but were late mannitol fermenters. The El Tor strains haemolysed sheep erythrocytes in tube, but the classical strains did not. The strains of both biotypes were of Heiberg group 1. The classical strains were phage type 3 and the El Tor type 4. A number of the El Tor strains were lysogenic, but none of the classical strains was positive for this characteristic.

No significant difference was observed between the growth curves for the classical and El Tor strains isolated in 1969 and 1982.

Preliminary studies of *in vitro* interactions between the classical and El Tor strains showed that, when grown together after inoculation with almost similar numbers of cells, both strains of the biotypes isolated in 1969 in Bangladesh grew rapidly up to 6 h, but the classical strain lagged by more than 1 log. By 8 h, the classical strain was completely overgrown by the El Tor (Table II). In contrast, the classical isolates from 1982 grew together at about equal rates, with the El Tor isolates of 1969 and 1982 for up to 24 h of observation. In one experiment, where the duration of observation was extended up to 50 days, a classical isolate of 1982 survived equally well in the presence of an El Tor isolate from 1969.

All the strains tested for toxigenicity by rabbit loop, skin PF, CHO and GM_1 ELISA assays, were positive. The mean PF titres of the classical and El Tor strains isolated in 1969-72 and 1979-82, indicated that strains of the former biotype were more potent toxin producers (Table III). The differences were more obvious for the 1979-82 isolates.

Discussion

It may be recalled that 1968-72 was a transitional period, in the sense that the classical biotype was slowly replaced by El Tor. During 1979-82, especially since September 1982, the reverse occurred.

TABLE I — WEEKLY ISOLATIONS OF *VIBRIO CHOLERAE* BIOTYPES CLASSICAL AND EL TOR FROM SEPTEMBER 3 TO DECEMBER 30, 1982 IN ICDDR,B HOSPITAL, DHAKA

Weeks	Classical			El Tor		
	Ogawa	Inaba	Total	Ogawa	Inaba	Total
03 Sep –09 Sep	0	19	19	31	65	96
10 Sep –16 Sep	0	38	38	27	44	71
17 Sep –23 Sep	0	68	68	29	23	52
24 Sep –30 Sep	0	66	66	18	37	55
01 Oct –07 Oct	0	88	88	43	41	84
08 Oct –14 Oct	0	117	117	54	48	102
15 Oct –21 Oct	0	137	137	91	51	142
22 Oct –28 Oct	0	130	130	71	65	136
29 Oct –04 Nov	0	113	113	77	101	178
05 Nov –11 Nov	0	129	129	85	58	143
12 Nov –18 Nov	1	273	274	121	70	191
19 Nov –25 Nov	1	286	287	109	76	185
26 Nov –02 Dec	0	304	304	88	54	142
03 Dec –09 Dec	0	236	236	74	23	97
10 Dec –16 Dec	0	202	202	40	14	54
17 Dec –23 Dec	0	231	231	28	7	35
24 Dec –30 Dec	1	180	181	12	6	18
	3	2617	2620	998	783	1781

TABLE II — *IN VITRO* INTERACTION BETWEEN *VIBRIO CHOLERAE* BIOTYPES CLASSICAL AND EL TOR USING ISOLATES OF 1969 AND 1982

Hours	Colony forming units per ml					
	Classical (1969)	El Tor (1969)	Classical (1982)[1]	El Tor (1982)	Classical (1982)	El Tor (1969)
0	0.6×10^1	0.7×10^1	1.9×10^2	9.0×10^1	9.0×10^1	1.1×10^1
4	1.0×10^3	3.9×10^3	4.9×10^5	1.3×10^5	3.2×10^4	2.1×10^4
6	3.0×10^3	3.8×10^4	ND[1]		ND	
8	0.0	1.3×10^6	4.0×10^8	5.5×10^8	7.2×10^8	4.1×10^8
12	0.0	1.5×10^8	1.7×10^8	6.0×10^8	8.0×10^8	6.0×10^8
24	0.0	1.5×10^9	4.0×10^8	2.3×10^9	9.5×10^8	1.2×10^9

[1] ND: not done

TABLE III — SKIN PF TITRES OF *VIBRIO CHOLERAE* BIOTYPES CLASSICAL AND EL TOR STRAINS ISOLATED DURING 1969-72 AND 1979-82

Biotype	Isolation period	Number of strains	Mean of PF titre (reciprocal)
Classical	1969-72	9	93.33
	1979-82	6	130.00
El Tor	1969-72	9	61.66
	1979-82	6	46.66

No significant difference has been observed to date with respect to the growth rate of individual classical or El Tor strains isolated in 1969 and 1982. However, competitive growth studies show that the earlier El Tor biotype had a definite growth advantage over the earlier classical biotype. Experiments carried out with the classical strains isolated in 1982 seem to indicate that the present classical biotype is different from those isolated in the Sixties as it can survive with the El Tor biotype of 1969 and 1982. However, the nature of change in the emergent classical strains has not yet been identified.

The biochemical characteristics (19) and the phage types (21) of strains belonging to both the biotypes isolated during the current epidemic are similar to those of the earlier classical and El Tor strains. These observations suggest that they might have originated in Bangladesh.

The classical and El Tor strains isolated during the current epidemic were all toxigenic by the rabbit loop, skin PF, CHO cell culture and GM_1 ganglioside assays. Titration of toxin produced by both the biotypes indicated that, under the same conditions of growth, the classical strains of 1979-82, were stronger producers of toxin than those of the El Tor biotype of 1969-72 and 1979-82.

TCBS medium has been considered very useful for isolation of the El Tor biotype (11) and is widely used throughout the world. However, little is known regarding its efficacy in isolating strains of classical biotype. In the present study, we were able to isolate equal numbers of *V. cholerae* strains of both the biotypes from TTGA (12) and TCBS media, indicating that TCBS is equally efficient in isolating *V. cholerae* strains regardless of their biotype.

The 1982 cholera epidemic in Bangladesh was caused by the re-emergence of the classical biotype of *V. cholerae*. This new classical biotype is able to compete with the El Tor and is more toxigenic in comparison to the prevailing El Tor biotype and may be indigenous to Bangladesh. A continuous surveillance system based on the biotyping of *V. cholerae* strains to monitor shifts between biotypes in cholera in endemic regions is valuable for a better understanding of the epidemiology of the disease.

Acknowledgements

This study was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). ICDDR,B is supported by countries and agencies

which share its concern about the impact of diarrhoeal diseases on the developing world. Current donors giving assistance to ICDDR,B are: AGFUND, Australia, Bangladesh, France, Japan, Saudi Arabia, Sweden, Switzerland, United Kingdom and USAID.

The authors are grateful to Dr. W.B. Greenough III for his helpful suggestions during the course of the study.

Correspondence and reprint requests should be addressed to: Dr. M.I. Huq.

References

1. Annapurna E, Sanyal SC. Enterotoxicity of *Aeromonas hydrophila*. *J Med Microbiol* 1977;10:317-23.
2. Bart KJ, Huq Z, Khan M, Mosley WH. Seroepidemiologic studies during a simultaneous epidemic of infection with El Tor Ogawa and classical Inaba *Vibrio cholerae*. *J Infect Dis* 1970;121(Suppl): S17-S24.
3. Basu S, Mukherjee S. Bacteriophage typing of Vibrio eltor *Experientia* 1968;24:299-300.
4. Cowan ST. Cowan and Steel's Manual for the identification of medical bacteria. Cambridge: University Press, 1974.238 p.
5. Craig JP. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. *Nature* 1965; 207:614-16.
6. De SN, Chatterje DN. Experimental study of mechanism of action of *Vibrio cholerae* on intestinal mucous membrane. *J Pathol Bacteriol* 1953;66: 559-62.
7. Dubey RS, Sanyal SC. Enterotoxicity of *Aeromonas hydrophila*: skin responses and in vivo neutralisation. *Zentralbl Bakteriol* (Orig A) 1978;242: 487-99.
8. Finkelstein RA, Mukerjee S. Hemagglutination: a rapid method for differentiating *Vibrio cholerae* and El Tor vibrios. *Proc Soc Exp Biol Med* 1963; 112:355-59.
9. Guerrant RL, Brunton LL, Schnaitman TC, Rebhun LI, Gilman AG. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. *Infect Immun* 1974;10:320-27.
10. Huq MI, Glass RI, Stoll BJ. Epidemiology of cholera. *N Engl J Med* 1980;303:643-44.
11. Kobayashi T, Enomoto S, Sakazaki R, Kuwahara S. A new selective isolation medium for the Vibrio group on a modified Nakanishi's medium (TCBS agar medium). *Jpn J Bacteriol* 1963;18:387-92.
12. Monsur KA. A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. *Trans R Soc Trop Med Hyg* 1961;55: 440-42.
13. Morris GK, Merson MH, Huq I, Kibrya AK, Black R. Comparison of four plating media for isolating *Vibrio cholerae*. *J Clin Microbiol* 1979;9:79-83.
14. Mukerjee S. Bacteriophage typing of cholera. *Bull WHO* 1963;28:337-45.
15. Mukerjee S. Diagnostic uses of cholera bacteriophages. *J Hyg (Camb)* 1961;59:109-15.
16. Richardson SH, Evans DJ, Jr. Isolation of cholera toxins by dextran sulfate precipitation. *J Bacteriol* 1968;96:1443-45.
17. Roy C, Mridha K, Mukerjee S. Action of polymyxin on cholera Vibrios: techniques of determination of polymyxin-sensitivity. *Proc Soc Exp Biol Med* 1965;119:893-96.
18. Sack DA, Huda S, Neogi PKB, Daniel RR, Spira WM. Microtiter ganglioside enzyme-linked immunosorbent assay for Vibrio and *Escherichia coli* heat-labile enterotoxins and antitoxin. *J Clin Microbiol* 1980;11:35-40.
19. Sakazaki R. Classification and characteristics of Vibrios. In: Principles and practice of cholera control. Geneva: World Health Organization, 1970; 33-37 (WHO public health papers no. 40).
20. Sanyal SC, Sakazaki R. Haemolysis test for *Vibrio cholerae*; an assessment of the various methods. *Indian J Microbiol* 1974;14:153-58.
21. Sanyal SC, Sil J, Dutta NK, Mukerjee S. Present status of classical cholera in India. *Indian J Med Res* 1972;60:1564-67.