JOURNAL OF CLINICAL MICROBIOLOGY, June 1985, p. 884–890
0095-1137/85/060884-07$02.00/0

Vol. 21, No. 6

In Vitro and In Vivo Cholera Toxin Production by Classical and El Tor Isolates of *Vibrio cholerae*

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Received 3 October 1984/Accepted 4 February 1985

A comparative study was carried out on the in vitro production of cholera toxin by 19 *Vibrio cholerae* El Tor isolates from patients with cholera in South Africa, one El Tor isolate from a patient in Malawi (a country approximately 1000 km north-northeast of South Africa), 6 El Tor and 12 classical type isolates from patients in Bangladesh, and 5 culture collection classical strains. Identical phage types and indistinguishable toxigenicities among the South African and Malawi *V. cholerae*, representing isolations obtained over a 10-year period, indicated that essentially a single strain was involved in the cholera of these regions. Similarly, phage typing and toxin profiles indicated that the 12 classical and 6 El Tor *V. cholerae* cultures in Bangladesh, all isolated in November 1983, represented just two strains. As assessed by titrations in Y-1 mouse adrenal and Chinese hamster ovary cell lines, the general order of toxigenicities was Bangladesh and culture collection classical > Bangladesh El Tor > southern African El Tor. The African isolates consistently gave rise to very low titers. Their relative reluctance to produce the toxin in vitro compared with the culture collection classical strains, particularly strain 569B, was confirmed by rocket electrophoresis. In somewhat of a contrast, maximum in vivo titers in rice water stools from cholera patients in South Africa and from both classical and El Tor type cholera patients in Bangladesh were essentially equal. It is postulated that under the continuous culture conditions that occur in vivo, cholera toxin concentrations can accumulate to a maximum level, depending on the rate of purging by the diarrheal fluid rather than the toxigenicity of the infecting strain. The relevance of these findings to the relative severities of classical and El Tor types of cholera is discussed.

Vibrio cholerae El Tor spread across India from the Far East in the 1960s, reaching the African continent in about 1970 (Fig. 1). Its arrival in the Republic of South Africa was anticipated in the early 1970s (15), and in 1974 the first epidemic of cholera in South Africa broke out in the western part of the province of Transvaal (15, 16, 21). Between 1974 and 1980, the disease was repeatedly introduced into Transvaal from the north but was kept under control by appropriate control measures coupled with good surveillance (14, 21). In 1980, however, the disease reached epidemic proportions, and since then cholera has been regarded as being endemic in Transvaal and Natal, another province of South Africa (16), with annual epidemics occurring each summer (Table 1).

The early 1970s and 1980s were also significant times in terms of cholera for Bangladesh. In 1973, the El Tor biotype was found to have completely replaced the classical biotype as the cause of endemic and epidemic cholera in that country (27). However, in 1979, 1980, and 1981, a few classical isolates were again detected (27), and since then the two biotypes have coexisted, thereby making possible various simultaneous comparative studies.

To microbiologists today, it is axiomatic that the symptoms of cholera result from the elaboration and action of cholera toxin, and both the nature of this enterotoxin and its mode of action in producing the characteristic choleraic diarrhea have been well characterized. It has been known for some time, however, that there is great variation in the ease with which cholera toxin production can be induced from different cholera isolates in the laboratory (8, 25, 26), but this variation and its possible relation to the pathogenesis of the disease appears to be a poorly studied area.

Evidence has been put forward that the classical type of cholera is a more severe disease than the El Tor form (1, 10). It is likely that this is attributable to virulence factor differences, although the generalization itself has not been fully verified (20), nor have the particular virulence factors involved been definitely identified. One possibility is that El Tor biotypes may be generally less enterotoxigenic than classical biotypes. This paper presents the results of a study on enterotoxigenicity of El Tor and classical strains of *V. cholerae*.

MATERIALS AND METHODS

Strains. The isolation histories of the *V. cholerae* strains used are summarized in Table 2. The bacteriophages and the phage typing method used were as described previously (22).

Media. Syncase sucrose (9) consisted of the following (per liter): 10 g of Casamino Acids (Difco Laboratories), 5.0 g of each of Na_2HPO_4, K_2HPO_4, and sucrose, 1.2 g of NH_4Cl, 0.09 g of Na_2SO_4, 0.042 g of $MgCl_2 \cdot 6H_2O$, 0.004 g of $MnCl_2 \cdot 4H_2O$, and 0.005 g of $FeCl_3 \cdot 6H_2O$. Syncase glucose consisted of the same formulation with sucrose replaced by glucose. The medium referred to as Casamino Acids-yeast extract (6) was syncase glucose with the following modifications: 20 g of Casamino Acids, 2.5 g of NaCl, no

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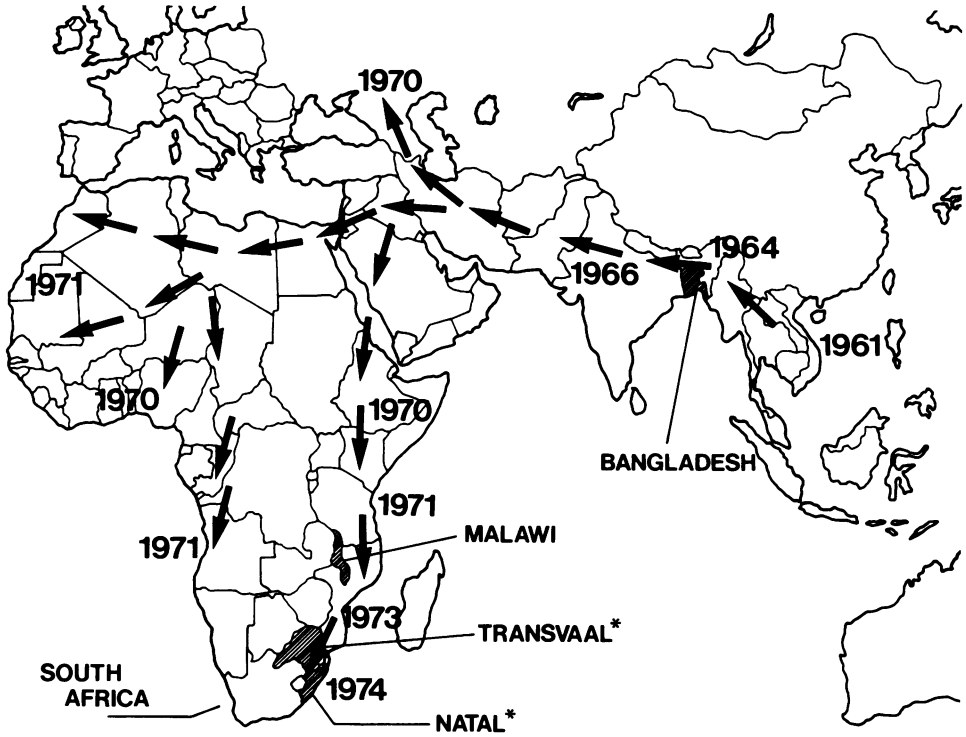

FIG. 1. The passage of *V. cholerae* El Tor into and through Africa. *, Provinces in the Republic of South Africa.

Na_2SO_4, and 6 g of yeast extract. These media were used at pH 7.3.

Culture and preparation for cholera toxin assay. Strains were initially grown in syncase sucrose broth, which is used routinely in the South African Institute for Medical Research (SAIMR) for assaying *Escherichia coli* heat labile enterotoxin in Y-1 mouse adrenal cell lines. When toxin was not detected in the culture filtrates (0.45-μm membrane filters) of South African El Tor isolates, filtrates of syncase glucose, brain heart infusion broth (Difco), and Casamino Acids-yeast extract broth cultures at 30 and 37°C held for various time periods, ranging from 18 to 48 h, were tried. Concentrated filtrates, filtrates of cultures in which the cells had been disrupted before filtration in an MSK homogenizer (Braun Melsungen AG, Melsungen, Federal Republic of Germany), and filtrates of cultures treated with polymyxin and mitomycin C by methods published previously (7, 17) were also tried.

As discussed below, the preparations judged to be optimal for comparative toxin tests were cell-free culture filtrates of brain heart infusion and Casamino Acids-yeast extract broth cultures grown in flasks on an orbital shaker (200 revolutions per min) at 30°C for 30 h. Volumes (8 ml) of the broths in 50-ml conical flasks were inoculated at the outset with single drops from brain heart infusion broth starter cultures.

Tests for active toxin. The systems maintained for routine assay of cholera and *E. coli* heat labile enterotoxins in the SAIMR and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB), were Y-1 and Chinese hamster ovary (CHO) cell lines, respectively. For the most part, therefore, tests for active toxin were done on Y-1 cells in the case of isolates submitted to the SAIMR and on CHO cells in the case of strains isolated at the ICDDRB. However, culture filtrates of each of the ICDDRB isolates were tested once on Y-1 cells to confirm the comparability of the two cell lines; the interchangeability was also confirmed with purified cholera toxin. Purified cholera toxin (Schwarz/Mann, Orangeburg, N.Y., in SAIMR tests; Sigma Chemical Co., St. Louis, Mo., in ICDDRB tests) was used as a positive control with each set of tests.

In Y-1 cell tests, wells in 96-well plastic tissue culture plates were seeded with 200 μl of Hams F10 medium containing approximately 10^6 Y-1 cells per ml; these were incubated under 5% CO_2 at 37°C. After 24 h, 50-μl volumes of doubling dilutions of the cell-free culture or stool filtrates were added in duplicate to the wells. Typical cytopathic effect was looked for after overnight incubation at 37°C under 5% CO_2.

The specificity of the cytopathic effect was confirmed with

TABLE 1. Cholera in South Africa[a]

Season	No. of bacteriologically confirmed cases	No. of treated cases	No. of deaths[b]
1974 (1st outbreak)	63	63	0
1980–1981 (cholera I)	3786	30,000 (E)[c]	42
1981–1982 (cholera II)	11,141	50,000 (E)	218
1982–1983 (cholera III)	7638	20,000 (E)	62

[a] Data for 1980 to 1983 reproduced from Epidemiological Comments, vol. 10, no. 10, 1983, Department of Health and Welfare, Pretoria, South Africa, by kind permission of H. G. V. Küstner, Director-General.
[b] Listed as "not all proven."
[c] E, Estimate.

TABLE 2. Histories and phage types of strains examined

Strain (no. of isolates)[a]	Isolation date (period)	Isolation source (no. of isolates)[b]	Biotype	Phage type[c]													
				I	II	III	IV	32	4	5	β	57	4996	13	14	16	24
MAL	1972	Malawi	El Tor Inaba	−	−	−	−	−	−	+	−	−	+	+	+	+	+
TEX	1973	Transvaal[d]	El Tor Inaba	−	−	−	−	−	−	+	−	−	+	+	+	+	+
Isolates (7)	1980–1981 (cholera I)	Transvaal	El Tor Inaba	−	−	−	−	−	−	+	−	−	+	+	+	+	+
Isolates (2)	1981–1982 (cholera II)	Transvaal	El Tor Inaba	−	−	−	−	−	−	+	−	−	+	+	+	+	+
Isolates (9)	1982–1983 (cholera III)	Transvaal (3), Natal (6)	El Tor Inaba	−	−	−	−	−	−	+	−	−	+	+	+	+	+
Isolates (12)	November 1983	Dhaka, Bangladesh	Classical Ogawa	+	−	±	+	−	−	−	+	−	−	−	−	−	−
Isolates (6)	November 1983	Dhaka, Bangladesh	El Tor Ogawa	−	−	−	−	−	−	+	+	−	−	−	−	−	−
569B	1953	India	Classical Inaba	+	+	+	+	−	−	−	+	+	−	+	−	+	+
NCTC 7254	1947	Egypt	Classical Inaba	+	+	+	+	−	−	−	+	+	−	+	−	+	+
NIH 35A3	pre-1942	India	Classical Inaba	+	+	+	+	−	−	−	+	+	−	+	−	+	+
NCTC 8050	pre-1949	India	Classical Ogawa	+	+	+	+	−	−	−	+	+	−	−	−	+	+
NIH 41	pre-1941	India	Classical Ogawa	+	+	+	+	−	−	−	+	+	−	+	−	+	+
A65716	1982	Transvaal, mine sewer	Ol Inaba	−	−	−	−	−	−	−	−	−	−	−	−	−	−
A7915	1983	Cape Province; river	Ol Ogawa	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London; NIH, National Institutes of Health; Bethesda, Md.
[b] Transvaal, Natal, and Cape Province are three provinces within the Republic of South Africa.
[c] From Lee and Furniss (22). +, >30 plaques at routine test dilution, i.e., the greatest dilution of phage giving confluent lysis on the propagating host; ±, <30 plaques.
[d] From a traveller who acquired it elsewhere in Africa or southern Europe.

neutralization controls. In these controls, 10 μl of cholera anti-toxin (Swiss Serum and Vaccine Institute, Berne, Switzerland) was added to 200 μl of cell-free culture or stool filtrate; after 30 min of incubation at 37°C, an additional 10 μl of antitoxin was added. After a second 15-min incubation period, these control samples were diluted and applied to the Y-1 cells in parallel with the untreated specimens. A reduction in titer of >1:64 in the neutralized line of wells, as compared with the non-neutralized lines, or complete neutralization in which the untreated specimen titers were already <1:64, was regarded as confirmation of the specificity of the cytopathic effect.

The CHO cell procedure differed slightly from that for Y-1 cells in that 40 μl of each dilution of cell-free culture or stool filtrate and the neutralization controls were added to 200 μl of freshly suspended CHO cells in Hams F12 medium with an approximate count of 10^6 CHO cells per ml of medium. Cytopathic effect was again looked for after overnight incubation at 37°C under 5% CO_2.

Rocket electrophoresis. Rocket electrophoresis was used as an alternative method of determining the levels of cholera toxin in cell-free culture and stool filtrates of the South African cultures and rice water stools. It was not possible to carry out this procedure on the Bangladesh cultures and stool specimens.

The proportion of cholera antitoxin to agarose found to be optimal for rocket electrophoresis was 75 μl of undiluted antitoxin to 15 ml of 1% agarose. Volumes (5 μl) of cell-free culture or stool filtrates concentrated 25 times in Minicon B-15 ultrafilters (Amicon Corp., Lexington, Mass.) were added to the wells before passing the current under a potential difference of 4 V/cm. Optimal dilutions of purified cholera toxin for standards were found to be 50, 25, 12.5, and 6.25 Lb dose units per 5 μl.

Toxin levels determined by this method are referred to as immunoprecipitated protein to distinguish them from levels of active toxin found in Y-1 and CHO cell titrations.

Rice water stools. Twenty-one rice water stool specimens were collected in the King Edward VII Hospital, Durban, Natal, South Africa, with the kind assistance of Y. Coovadia, Department of Microbiology. These specimens were taken from bed pans with special care to avoid contamination with urine and were held frozen at −20°C until just before the toxin assays. They were then thawed, centrifuged at approximately 3000 × *g* for 30 min in a refrigerated centrifuge, and filtered through 0.45-μm membrane filters. An additional four specimens were submitted by the Hillbrow Hospital, Johannesburg, South Africa, and were centrifuged and filtered immediately.

In ICDDRB, 18 rice water stool specimens were collected with an anal catheter in the Centre's Hospital and were immediately centrifuged and membrane filtered as before. The filtrates were held frozen at −20°C pending toxin assay and bacteriological confirmation of the diagnosis of cholera.

A proportion of the *V. cholerae* isolates from the South African stools and all those from the Bangladesh stools were phage typed and tested for their in vitro enterotoxigenicity as described above.

TABLE 3. Y-1 and CHO cell line titrations of cell-free culture filtrates[a]

Strain[b]	Biotype	No.of times tested	Mean titer (Y-1 or CHO)[c]	Highest titer	
				Titer[c]	PCTE[d] (pg/ml)
S. African	El Tor	21	4	32	1×10^3
Bangladesh	El Tor	10	32	512	2×10^4
Bangladesh	Classical	14	4096	8192	3×10^5
569B	Classical	8	128	512	2×10^4
NCTC 7254	Classical	6	1024	8192	3×10^5
NCTC 8050	Classical	6	512	2048	7.5×10^4
NIH 35A3	Classical	2	64	64	2.5×10^3
NIH 41	Classical	2	128	128	5×10^3

[a] Brain heart infusion and Casamino Acids-yeast extract broth cultures at 30°C.

[b] NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London; NIH, National Institutes of Health, Bethesda, Md.

[c] Expressed as the reciprocal of the dilution.

[d] PCTE, Purified cholera toxin equivalent (approximate) based on an end point obtained with purified cholera toxin at a dilution equivalent to ±35 pg/ml.

RESULTS

Phage typing. The same phage typing pattern was exhibited by all the southern African isolates (Table 2), indicating that it was essentially a single strain that entered South Africa in 1974 and became responsible for the subsequent epidemics of cholera that occurred there. Two isolates, strains A65716 and A7915 (Table 2), were found with unusual biotypes; because they were nontypable with the typing bacteriophages, strongly hemolytic, and apparently not toxigenic, these were considered to be further representatives of the increasingly well-recognized group of environmental O1 *V. cholerae* that lack the gene for toxin production (18).

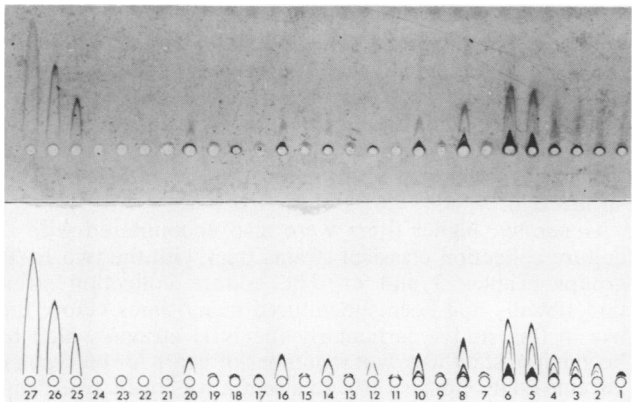

FIG. 2. Rocket electrophoresis of brain heart infusion broth *V. cholerae* culture filtrates. Wells contain the following: wells 1 through 6, strain 569B at 1×, 2×, 5×, 10×, 25×, and 25× (different day's preparation) concentrations, respectively; wells 7 and 8, NCTC 8050 at 1× and 25×, respectively; wells 9 and 10, MAL at 1× and 25×, respectively; wells 11 through 20, alternate 1× and 25× culture concentrates of five South African isolates, respectively; wells 21 and 22, 1× and 25× isolate, respectively, thought to be *V. cholerae* but subsequently identified as *Enterobacter cloacae* (consistently negative); wells 23 and 24, 1× and 25× BHI broth control, respectively; wells 25 through 27, 0.05, 0.1, and 0.2 mg of purified cholera toxin per ml, respectively.

The Bangladesh strains used were isolated over a period of 2 days. As judged by phage typing (Table 2), the 18 isolates represented just two strains, with one being biotype El Tor and the other being a classical biotype. The typing of additional isolates not used in this study indicated that other strains were in circulation.

Active toxin. Cholera toxin activity was not detected or was only detected spasmodically and feebly in Y-1 cells exposed to cell-free culture filtrates of the southern African isolates cultured in syncase sucrose and syncase glucose at 30 and 37°C or in brain heart infusion broth at 37°C for 24 or 48 h. Mechanical disruption of the *V. cholerae* cells and mitomycin C and polymyxin toxin release procedures did not alter this finding. The control in these tests was strain 569B, which was strongly positive in the simple cell-free culture filtrates, with or without being concentrated, and after mechanical disruption but was negative after polymyxin and mitomycin release procedures. These results were checked and confirmed by ligated rabbit ileal loop tests. The Bangladesh strains were not tested by any of these methods.

The media and culture conditions ultimately adopted for subsequent tests were brain heart infusion and Casamino Acids-yeast extract broths incubated at 30°C for 30 h. These media and conditions yielded consistent, although low, titers in Y-1 cells with the African cholera isolates and gave rise to the highest titers obtained in this study with strain 569B. The final analysis showed that the titers of brain heart infusion broth cultures were consistently two- to fourfold higher than those of Casamino Acids-yeast extract cultures.

Titers obtained with the African El Tor cultures remained consistently low and were generally about 1:4. The highest titer recorded, taken to represent the in vitro toxigenic potential of the isolates in the systems used, was 1:32. This was the mean titer obtained with the Bangladesh El Tor isolates, although on one occasion a titer of 1:512 was recorded in the assays on these (Table 3). Generally, higher mean and maximum titers were found with both culture collection and freshly isolated classical strains.

Based on an end point obtained in Y-1 and CHO cell lines with purified cholera toxin, a conversion of the highest titers obtained with the different strains to picograms per milliliter is included in Table 3.

Toxin levels as immunoprecipitated proteins. A total of 56 cell-free culture filtrate preparations from 12 of the African isolates and 16 preparations from 4 of the culture collection classical strains were assayed by rocket electrophoresis. As with Y-1 titrations, consistent toxin profiles were produced by the different African isolates (Fig. 2). The in vitro toxigenicities, as represented by the highest toxin levels

TABLE 4. Levels of cholera toxin in cell-free culture filtrates[a] as determined by rocket electrophoresis

Strain	Biotype	No. of times tested	Highest titer (PCTE[b] [μg/ml)
South African	El Tor	56	3.2
569B	Classical	10	26.0
NCTC 7254	Classical	3	21.0
NCTC 8050	Classical	2	16.0
NIH 41	Classical	1	6.8

[a] Brain heart infusion and casamino acids-yeast extract broth cultures at 30°C.

[b] PCTE, Purified cholera toxin equivalent; based on standards prepared with purified cholera toxin expressed as micrograms per milliliter of immunoprecipitated protein.

found, are given in Table 4 and are expressed as purified cholera toxin equivalents of immunoprecipitated protein.

Although the proportions involved are different, these results generally support the findings obtained with Y-1 assays that the African El Tor isolates produced lower in vitro levels of toxin than the classical strains with which they were compared.

Toxin levels in rice water stools. Of the 25 South African and 18 Bangladesh rice water stools examined, 34 gave cytopathic effect in Y-1 or CHO cells deemed due to cholera toxin and which was neutralized with antitoxin. Of the remaining nine filtrates (all South African stools), eight were negative and one exhibited uncharacteristic cytopathic effect in the tissue culture cells.

Again, the highest titer found was considered the most important feature in that it represented levels to which toxin concentrations could definitely rise in the stools of a patient. The highest titer noted in the South African cholera (El Tor) and the Bangladesh classical cholera stools was 1:512, and that in the Bangladesh El Tor stools was 1:256 (Table 5). Thus, the maximum levels of cholera toxin found in patient stools was comparable in each of the three cholera categories.

A comparison is made in Table 6 between in vivo maximum levels and the maximum titers in vitro obtained in cell-free culture filtrates of broth cultures of *V. cholerae* strains isolated from these stools.

Rocket electrophoresis was only carried out on the South African stools. Some anomalies resulting from the development of multiple rockets occurred with these filtrates, but it was considered that bands corresponding in form and density to the purified cholera toxin standards could be distinguished from others that were present. Based on these comparisons with the standards, the maximum concentration of immunoprecipitated protein found in any of the stool specimens was 10 μg/ml; this compares with in vitro levels of <1 μg/ml in the cell-free culture filtrates of the corresponding *V. cholerae* isolates (Table 6) and a highest level of 3.2 μg/ml found at any time with any of the southern African isolates.

DISCUSSION

The carefully monitored arrival and establishment of El Tor cholera in South Africa over the past decade and the recent development of a coexistence by classical and El Tor biotypes in Bangladesh have provided novel opportunities to examine the epidemiology and pathogenesis of cholera to previously unattainable depths.

The 20 *V. cholerae* isolates submitted for phage typing from cases of cholera in southern Africa (Malawi, 1 isolate; South Africa, 19 isolates) all exhibited the same phage typing pattern. All except those labeled MAL and TEX and the six listed in Table 2 as originating from Natal, South Africa, were selected at random from a large bank of available isolates. The isolates from Natal were six of the strains isolated from the rice water stools collected in Durban, South Africa, for cholera toxin assay. The implications, therefore, are that cholera in South Africa has resulted from the spread of a single strain of this phage type.

TABLE 5. Cholera toxin levels in rice water stools

Origin	Biotype	No. of stool specimens examined	Y-1 or CHO titers		Rocket electrophoresis range (PCTE[b] [μg/ml])
			Range[a]	Peak PCTE[b] (pg/ml)	
South Africa	El Tor	25	0–512	2×10^4	0–10
Bangladesh	El Tor	6	8–256	1×10^4	ND[c]
Bangladesh	Classical	12	0–512	2×10^4	ND

[a] Expressed as the reciprocal of the dilution.
[b] PCTE, Purified cholera toxin equivalent (approximate) based on an end point and standards as described in Tables 3 and 4.
[c] ND, Not done.

TABLE 6. Comparison of maximum in vivo and in vitro levels of cholera toxin found

Origin	Biotype	Y-1 or CHO titers[a]		REP[b] (μg/ml)	
		Stools	CFCF[c]	Stools	CFCF[c]
South Africa	El Tor	512	32	10	≤1
Bangladesh	El Tor	256	512	ND[d]	ND
Bangladesh	Classical	512	8192	ND	ND

[a] Expressed as the reciprocal of the dilution.
[b] REP, Rocket electrophoresis.
[c] CFCF, Cell-free culture filtrates on brain heart infusion and Casamino Acids-yeast extract cultures at 30°C.
[d] ND, Not done.

The existence of the same phage type in southern African states has been noted previously in an examination of 1135 strains of *V. cholerae* biotype El Tor from Africa, Asia, Europe, and the United States (22). The southern African type was uncommon elsewhere but differed in only one phage character (β) from the most common type found, and it may be descended from that phage type.

Phage typing similarly indicated that the 12 classical cholera isolates from cases in Bangladesh again probably represented a single strain and the 6 El Tor isolates represented a second single strain (Table 2). Neither phage pattern was encountered frequently in the previous study (22). Phage typing of other case isolates not included in this study showed that other strains were in circulation in Bangladesh at the time in addition to these two (13; J. V. Lee, unpublished data).

In all three groups, the southern African El Tor and the Bangladesh classical and El Tor, the similar in vitro toxigenicities of the different isolates within each group supported the concept that each group represented a single strain. In terms of Y-1 and CHO cell titers, the order of toxigenicities was Bangladesh classical > Bangladesh El Tor > southern African El Tor. This is in accord with a previous observation that classical strains were more choleragenic than El Tor strains (25).

Generally, higher titers were also encountered with the culture collection classical strains than with the two El Tor groups (Tables 3 and 4). The culture collection strains undoubtedly had been subcultured many times before their use in this study, particularly the NIH strains which had been in constant use over a number of years for biotype and vaccine control purposes. No attempt was made to enhance their toxigenicities by animal passage (2, 5, 25). Strain 569B has an extensive history of use in cholera toxin studies and production; it is probable that the descendant utilized in this study was relatively atoxigenic compared with its original passaged ancestor (5).

It is not a new observation that cholera isolates may not readily yield enterotoxin when grown in vitro (8, 25, 26), but there appears to be little direct data on how in vitro and in vivo toxigenicities may be related, although an attempt was made at an early stage in cholera toxin research "roughly to quantitate" the toxin in stools of cholera patients (4). Given

that the highest titers recorded in the present study represent the minimum toxigenicities of the strains (i.e., the strains showed themselves to be able to produce cholera toxin at least to those levels), it is of interest that, although the in vitro toxigenicities of the three groups of strains varied quite markedly, there was little apparent difference between their in vivo toxigenicities (Table 6). Also of interest are the maximum in vitro-to-in vivo titer ratios of 32:512, 512:256, and 8192:512 for the South African El Tor, Bangladesh El Tor, and Bangladesh classical cases, respectively.

Rocket electrophoresis provided immunological support for the findings by the tissue culture approach of marked differences between in vitro toxigenicities among southern African El Tor and the culture collection classical strains (Table 4) and between the in vitro and in vivo toxigenicities of the South African isolates (Table 6). Unfortunately, rocket electrophoresis could not be carried out during that part of the study that took place in Bangladesh, and comparisons by this method between the three cholera groups were not possible. Multiple rockets, particularly with stool filtrates, highlighted the inadequacies of the antisera used. More refined antibody preparations should now be possible and should permit further studies in greater depth on in vivo toxin production by rocket electrophoresis, enzyme-linked immunosorbent assays, or other suitable assay systems.

The precise relationship between the levels of active toxin as determined by tissue culture and the levels of immunoprecipitated protein found with rocket electrophoresis is uncertain. The dilution of purified cholera toxin at which approximately 50% of Y-1 cells rounded up was equivalent to a concentration of approximately 35 pg/ml. Based on this value, the stool-to-culture filtrate maximum titers of 512:32 in the South African tests (Table 6) represented a 16-fold difference in concentration compared with approximately a 10-fold difference in concentrations as determined by rocket electrophoresis. The immunoelectrophoretic technique measures some form of total protein, whereas tissue culture estimations reflect the number of toxin molecules required to activate adenylate cyclase in a eucaryotic cell. Measurable activation has been reported to occur with as few as 10 toxin molecules (23), or even just one (12) molecule, per cell. Beyond this, no definite statement seems possible about the relationship between the absolute values. The values are widely discrepant, with rocket electrophoresis values most readily expressed in micrograms per milliliter and Y-1 and CHO cell estimations in picograms per milliliter (Tables 3 through 5).

By our estimation, the specific activity of purified cholera toxin (35 pg/ml) is in reasonable accord with previous estimations of 28 pg/ml (11) and 15 pg/ml (3). The peak in vitro toxigenicities of 1 and 20 ng/ml recorded here with the southern African and Bangladesh El Tor isolates, respectively, compares favorably with a value of 7.5 ng/ml associated with an El Tor isolate from a patient in Texas (19).

On the basis of case-to-infection ratios (10) and case fatality rates (1), it has been proposed that cholera from classical strains is generally more severe than that from El Tor strains. In a more recent study in Bangladesh (20), case fatality rates were not significantly different for the two types. In the well-circumscribed and carefully monitored 1974 mine outbreak of El Tor cholera in South Africa (Table 1; 15), the case-to-infection ratio (57% of infected persons exhibited symptoms of cholera) was, in fact, higher than that reported for classical strains by Gangarosa and Mosley (10). Although this probably reflects, at least in part, a lack of previous exposure and immunity within the mine community, it could also indicate that it may not be possible to generalize about the virulence of classical and El Tor cholera. As far as toxin as a virulence factor is concerned, this appears to find support in the observation reported here of similar maximum toxin levels found in rice water stools from South African El Tor, Bangladesh El Tor, and Bangladesh classical type cases.

In contrast, it does seem to be a fair generalization, and one supported by the present findings, that classical strains appear to lend themselves more readily than El Tor strains to in vitro cholera toxin production. One possible explanation for the different toxigenicities found within and between the two groups may lie in the recent observation (24) that classical strains all contain a nontandem chromosomal duplication of the *ctx* (cholera toxin) operon, whereas about 70% of El Tor strains have only a single copy of *ctx*, and the remainder possess two or more *ctx* copies present on a tandemly repeated genetic element. A study aimed specifically at examining toxigenicity in relation to the number of *ctx* gene copies in different strains is desirable. It is difficult to see how the enhancement of choleragenicity by animal passage can be explained simply in terms of numbers of gene copies, and it may be that other virulence factors are also involved in phenotypic toxigenicity.

Possibly, the difference between the in vivo and in vitro situations is that under the continuous culture conditions that occur in vivo, toxin concentrations can accumulate with time to similar levels, regardless of the relative toxigenicities of different infecting strains. The peak level reached is probably a function of the purging rate by the diarrheal fluid rather than the toxigenicity of the *V. cholerae* involved. By this theory, however, one would expect incubation times to be shorter in infections with more toxigenic strains; this perhaps merits a complete study.

Alternatively, in vitro differences may simply be a function of differing growth characteristics in artificial media and the result of failure so far to find suitable universal media and conditions for toxin production. Certainly, the culture conditions of maximal aeration at 30°C, generally accepted as those best for in vitro cholera toxin production, are far removed from in vivo reality, suggesting that this alternative may be partially relevant.

Quantitative measurements of *V. cholerae* levels in the media were abandoned early in the study when it became apparent that the available counting methods were not sufficiently sensitive to detect genuine strain differences in in vitro growth ability. Under the conditions of collection, freezing, and transport of the South African stool specimens, fecal *V. cholerae* counts were impracticable, and such counts were not done in the Bangladesh section of the study either. An examination of *V. cholerae* levels in stools may be a useful adjunct to further comparative studies on classical and El Tor cholera.

ACKNOWLEDGMENTS

We thank Y. Coovadia, Department of Microbiology, Faculty of Medicine, University of Natal, Durban, South Africa, for collection of rice water stool specimens and cultures from cholera cases in South Africa and H. G. V. Küstner, Director-General, Department of Health and Welfare, Pretoria, South Africa, for assistance with the epidemiological data for Table 1. We are grateful to W. B. Greenough III, Director, and the Board of Trustees, ICDDRB, for making possible the consultancy during which that part of the study done in Bangladesh was carried out. Most of the work for this project was directed by the senior author during a 2-year period of research at the South African Institute for Medical Research and a

1-month consultancy at the International Centre for Diarrhoeal Disease Research, Bangladesh.

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