Epidemiol. Infect. (1995), **114**, 65–70

Distribution and virulence of *Vibrio cholerae* belonging to serogroups other than O1 and O139: A nationwide survey

A. K. MUKHOPADHYAY[1], P. K. SAHA[1], S. GARG[1], S. K. BHATTACHARYA[1], T. SHIMADA[2], T. TAKEDA[3], Y. TAKEDA[4] AND G. BALAKRISH NAIR[1]

[1]*National Institute of Cholera and Enteric Diseases, Beliaghata, Calcutta 700 010, India*

[2]*Department of Bacteriology, National Institute of Health, Shinjuku-ku, Tokyo 162, Japan*

[3]*Department of Infectious Diseases Research, National Children's Medical Research Center, Setagaya-ku, Tokyo 154, Japan*

[4]*International Medical Center of Japan, Shinjuku-ku, Tokyo 162, Japan*

(*Accepted 2 October 1994*)

SUMMARY

The distribution and virulence of *Vibrio cholerae* serogroups other than O1 and O139 in India before, during and after the advent of O139 serogroup was investigated. A total of 68 strains belonging to 31 different 'O' serogroups were identified during the study period. With the exception of O53, there was no spatial or temporal clustering of any particular non-O1 non-O139 serogroup at any given place. Two of the 68 strains examined produced cholera toxin (CT) which could only be partially absorbed with anti-CT immunoglobulin G. Tissue culture assay revealed that some of the non-O1 non-O139 strains produced factors which evoked either a cell rounding or cell elongation response depending upon the medium used. This study indicates that serogroups other than O1 and O139 should also be continuously monitored.

INTRODUCTION

The disease cholera is caused by *Vibrio cholerae* belonging to two different somatic serogroups, O1 and O139; the latter serogroup being a recent addition [1]. *V. cholerae* O139 has widely been proclaimed as the eighth pandemic strain of cholera [2]. Since its genesis in late 1992 [3, 4], the origin of the O139 serogroup and its relatedness to the O1 serogroup has been much debated. While the genetic elements relating to cholera toxin and other putative toxins in the virulence cassette and the genes regulating the expression of these toxins of the O139 serogroup resemble that of the O1 Eltor biotype [5], the chemical composition of the lipopolysaccharide of the O139 serogroup [6] as well as the serological reactivity [7] places these organisms as a distinct entity. That O139 serogroup is

* Corresponding author: Dr G. Balakrish Nair, Assistant Director, National Institute of Cholera and Enteric Diseases, Beliaghata, Calcutta 700 010, India.

not a simple mutant of O1 Eltor, is evident from the fact that the O139 serogroup lacks all the genes required for the synthesis of O1 antigen and for the Ogawa modification [8, 9].

In the span of one year, the O139 serogroup has spread into all cholera endemic areas in India [10, 11] and to several countries in South Asia [12]. With the advent of the O139 serogroup, the non-O1 serogroups of *V. cholerae*, previously relegated to negligible clinical significance, have acquired importance. The fact that the O139 serogroup, non-O1 serogroup, has so suddenly and explosively emerged as the causative agent of cholera would indicate the possibility of the recurrence of such an event. This study was performed to understand the spatial and temporal distribution of *V. cholerae* serogroups other than O1 and O139 in India before, during and after the advent of O139 serogroup and to analyse the virulence determinants associated with these strains.

MATERIALS AND METHODS

Source of strains

Being the National Reference Laboratory of Cholera in India, strains of *V. cholerae* from different parts of the country are submitted to the National Institute of Cholera and Enteric Diseases (NICED) for identification, serogrouping and phage typing. In addition, a constant surveillance to assess variations in serogroups of *V. cholerae* isolated from hospitalized patients with acute diarrhoea at the Infectious Diseases Hospital (IDH) has been underway at the NICED for the past several years. The data for this report included strains of *V. cholerae* non-O1 non-O139 isolated or received from March 1992 to December 1993.

Bacteriology and serology

Strains of *V. cholerae* isolated from patients admitted with acute diarrhoea at the IDH, Calcutta and strains received from different parts of India were isolated and identified using procedures described in detail elsewhere [13, 14]. Strains that did not agglutinate with O1 and O139 antisera were further serogrouped by the somatic O antigen serogrouping scheme of *V. cholerae* developed at the National Institute of Health, Tokyo, Japan [15].

Detection of cholera toxin-like toxin

Strains were grown in Casamino Acid–Yeast Extract (CAYE) supplemented with 90 μg/ml lincomycin in static condition at 30 °C. The culture supernates of all the *V. cholerae* non-O1 non-O139 strains were examined for the presence of cholera toxin (CT)-like enterotoxin using a highly sensitive bead-ELISA [16]. Various dilutions of purified CT (Sigma Chemical Co., Mo, USA) and uninoculated medium (negative control) were run concurrently whenever a batch of the culture supernate of the test strains were assayed by the bead-ELISA.

Absorption assay with anti-CT IgG

An absorption assay was performed by bead-ELISA to determine whether CT present in culture supernates of strains yielding a positive result by the CT bead-ELISA could be absorbed with anti-CT IgG. In this assay, culture supernate of the

CT-producing strain was pre-incubated at 37 °C for 1 h with 10 μg of rabbit anti-CT IgG/ml. From earlier experiments it was determined that 10 μg of anti-CT IgG/ml can completely absorb 500 ng of pure CT/ml [17]. Subsequently, the coated beads were introduced into 0·5 ml of the above described incubation and the bead-ELISA was performed as described previously.

Tissue culture assay

All the strains were grown in Trypticase Soy Broth (TSB, Difco) supplemented with 0·6% yeast extract (Difco) and in AKI medium (Bacto peptone 1·5%; yeast extract 0·4%; NaCl 0·5%; $NaHCO_3$ 0·3%; pH 7·4) in shaking condition for 18 h. Cultures were centrifuged at 6000 r.p.m. for 15 min (Sorvall RT6000B, DuPont, USA) and the supernate was passed through 0·22 μm filter unit (Millex-GS, Millipore, USA), collected in sterile test tubes and kept at 4 °C until used.

CHO and HeLa cells were grown as monolayer in Dulbecco's Minimum Essential Medium (Nissui Pharmaceutical Co. Ltd., Japan) supplemented with 10% (vol/vol) horse serum (Gibco Laboratories, USA). Cell lines were maintained in tissue-culture flasks (25 cm^2) at 37 °C in a humidified CO_2 5% atmosphere. A confluent monolayer of CHO and HeLa cells grown for 3–4 days was removed from the tissue culture flasks and 200 μl of the cell suspension ($6{\cdot}4 \times 10^3$ cells) was added to each of the 96 well plates along with 50 μl of the cell-free culture filtrate and incubated as described above. Cytotonic and cytotoxic changes were recorded at 24 h and 36 h. For controls, wells received the uninoculated culture medium as negative control and pure cholera toxin as positive control.

RESULTS

During the period of study, a total of 68 strains of *V. cholerae* non-O1 non-O139 were examined. Of these, 36 were isolated from patients with acute diarrhoea admitted to IDH, Calcutta while the remaining 32 strains were received from various parts of India as shown in Table 1.

A total of 31 different O serogroups which represents 20% of the currently recognized 155 'O' serogroups of *V. cholerae* (18) were identified in this study. Three strains could not be typed while three strains were rough. With the exception of O53, there was no spatial or temporal clustering of any particular non-O1 and non-O139 serogroup at any given place. The eight O53 strains of *V. cholerae* were received in a single batch of 88 cultures recovered from an outbreak of cholera which occurred in Madurai, South India, in November 1992 during the first O139 cholera epidemic in that city.

Of the 68 strains examined, only 2 of the 3 strains of *V. cholerae* belonging to the serogroup O74 produced cholera-like toxin when examined by the bead-ELISA. The toxin produced by both the strains could only be partially absorbed with anti-CT IgG in the absorption assay by the bead-ELISA.

Examination of the sterile culture filtrates of the 68 strains grown in two different media namely TSB and AKI yielded interesting results when examined in CHO and HeLa cells (Table 2). Among the strains which evoked a response in CHO and HeLa cells, it was clear that strains grown in TSB more often produced morphological changes of CHO and HeLa cells consistent to that of cell

Table 1. *Distribution of various non-O1 non-O139 serogroups of* V. cholerae *at various places in India isolated from March 1992 to December 1993*

Place	Serogroup (No. of strains)
Calcutta	O2(3), O5(2), O6(1), O7(1), O10(1), O24(1), O32(2), O34(1), O36(1), O37(2), O39(1), O43(1), O45(1), O53(1), O54(3), O56(2), O60(1), O69(2), O74(3)*, O134(1), O145(1), Rough(1), OUT(3)
Madras	O7(1), O14(1), O26(1), O69(1), Rough(2)
Vellore	O37(2)
Madurai	O45(1), O47(1), O53(8)
Nagpur	O5(1), O7(2), O8(1), O64(1), O68(1), O104(1)
Pune	O5(1), O10(1), O27(1)
Aurangabad	O71(1)
Warangal	O12(1)
Jodhpur	O27(2)

* Two of the three strains belonging to serogroup O74 of *V. cholerae* non-O1 non-O139 produced cholera toxin as determined by the Bead-ELISA.

Table 2. *Response of CHO and HeLa cells to culture filtrates of* V. cholerae *non-O1 non-O139 examined in the study*

	Number of strains			
	CHO cells		HeLa cells	
Morphological change	TSB	AKI	TSB	AKI
Unaffected	37	44	47	47
Cytotoxic	4	8	8	0
Cell-rounding	2	14	0	13
Cell elongation	25	2	13	8

elongation. In contrast, when strains were grown in AKI medium, a cell rounding response was observed more often in CHO and HeLa cells (Table 2).

DISCUSSION

Strains of *V. cholerae* belonging to serogroups other than O1 and O139, broadly classified as non-O1 non-O139, can no longer be ignored. A case in point is the sudden emergence of a non-O1 serogroup, currently classified as O139 Bengal, which has necessitated the need to continuously monitor the non-O1 serogroups of *V. cholerae*. It may not be prudent to draw a line of distinction between serogroups like O1 and O139 and the other serogroups considering the recent finding that serogroups O22 and O155 possess somatic (O) antigen factors in common with *V. cholerae* O139 [18].

In this study, there was no temporal clustering of serogroups apart from O53 isolated in Madurai, South India. Tissue culture studies on the culture filtrates of the O53 strains indicated that the strains do not represent a single clone because of their different responses on CHO and HeLa cells. Three classes of strains of non-O1 non-O139 could be recognized in this study based on their virulence factors. This included strains which produced a cholera-like toxin, strains which did not produce cholera-like toxin but evoked distinct changes in tissue culture cell lines

and strains which neither produced a cholera-like toxin or affected CHO and HeLa cells.

The CT gene has recently been found to be associated with a virulence cassette DNA fragment carrying a core region which includes the *cep* (core encoded pilus), *ace* (accessory cholera enterotoxin) and *zot* (zona occludens toxin) genes associated with colonization and pathogenesis of *V. cholerae* [19–21]. Further, it has also been documented that most strains of *V. cholerae* independent of its serogroup carry the *ToxR* gene which encodes a regulatory protein known to coordinately regulate virulence attributes including cholera toxin [22, 23]. The two O74 *V. cholerae* strains which produced CT-like toxin represent strains which may have the potential to flare into epidemics given the right set of circumstances and such strains need to be closely watched.

In this study, the clinical profile of patients from whom the 36 strains of *V. cholerae* non-O1 non-O139 were isolated from IDH, Calcutta was indistinguishable from full-blown cholera. Had the serogroup not been identified, it would be difficult for the clinician to distinguish these cases from cholera. Therefore, the non-O1 non-O139 serogroups represent an intriguing group of strains which can cause a typical cholera like disease but which do not produce cholera toxin and presumably the other putative toxins associated with serogroups O1 and O139. Despite deletion of all known putative toxin genes in the live oral attenuated vaccine strain (CVD 110), it was observed that CVD 110 was still capable of evoking residual diarrhoea in human volunteers [24]. Majority of the non-O1 non-O139 strains investigated in this study, like the vaccine strain, do not produced cholera toxin and presumably the other toxins associated with the virulence cassette but yet cause a cholera-like disease. These non-O1 non-O139 strains represent important strains which would enable us to bridge the gap in our understanding of what causes the residual diarrhoea in strains which do not possess any of the currently recognized virulence factors.

ACKNOWLEDGEMENTS

The award of a Junior Research Fellowship to P. K. Saha from the Council of Scientific and Industrial Research (CSIR), New Delhi, India, is gratefully acknowledged.

REFERENCES

1. Bhattacharya SK, Bhattacharya MK, Nair GB, et al. Clinical profile of acute diarrhoeal cases infected with the new epidemic strains of *V. cholerae* O139: Designation of the disease as cholera. J Infect 1993; **27**: 11–5.
2. Swerdlow DL, Ries AA. *Vibrio cholerae* non-O1 – the eighth pandemic? Lancet 1993; **342**: 382–3.
3. Ramamurthy T, Garg S, Sharma R, et al. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet 1993; **341**: 703–4.
4. Albert MJ, Siddique AK, Islam MS, et al. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. Lancet 1993; **341**: 704.
5. Walder MK, Mekalanos JJ. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect Immun 1994; **62**: 72–8.
6. Hisatsune K, Kondo S, Isshiki Y, Iguchi T, Kawamata Y, Shimada T. O-antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. BBRC 1993; **196**: 1309–15.

7. Shimada T, Nair GB, Deb BC, Albert MJ, Sack RB, Takeda Y. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet 1993; **341**: 1347.
8. Manning PA, Strocher UH, Morona R. Molecular basis for O-antigen biosynthesis in *Vibrio cholerae* O1: Ogawa–Inaba switching. In: Wachsmuth IK, Blake PA, Osvik O, eds. *Vibrio cholerae* and cholera: Molecular to global perspectives. Washington: American Society for Microbiology, 1994: 77–94.
9. Johnson JA, Salles CA, Panigrahi P, et al. *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* ElTor but has important differences. Infect Immun 1994; **62**: 2108–10.
10. Garg S, Saha PK, Ramamurthy T, et al. Nationwide prevalence of the new epidemic strain of *Vibrio cholerae* O139 Bengal in India. J Infect 1993; **27**: 108–9.
11. Nair GB, Ramamurthy T, Bhattacharya SK, et al. Spread of *Vibrio cholerae* O139 Bengal in India. J Infect Dis 1994; **169**: 1029–34.
12. World Health Organization. Cholera-update, end of 1993. Weekly Epidemiol Rec 1994; **69**: 13–7.
13. Ramamurthy T, Bag PK, Pal A, et al. Virulence patterns of *Vibrio cholerae* non-O1 strains isolated from hospitalised patients with acute diarrhoea in Calcutta, India. J Med Microbiol 1993; **39**: 310–7.
14. Nair GB, Ramamurthy T, Garg S, Takeda T, Takeda Y. Characteristics of *Vibrio cholerae* isolated from hospitalized patients with acute diarrhoea in Calcutta, India: A four year analysis. Lab Medica Intl 1993; **X**: 29–33.
15. Shimada T, Arakawa E, Itoh K, et al. Extended serotyping scheme for *Vibrio cholerae*. Curr Microbiol 1994; **28**: 175–8.
16. Oku Y, Uesaka Y, Hirayama T, Takeda Y. Development of a highly sensitive Bead-ELISA to detect bacterial protein toxins. Microbiol Immunol 1988; **32**: 807–16.
17. Ramamurthy T, Bhattacharya SK, Uesaka Y, et al. Evaluation of the bead enzyme linked immunosorbent assay for detection of cholera toxin directly from stool specimens. J Clin Microbiol 1992; **30**: 1783–6.
18. Shimada T, Arakawa E, Itoh K, et al. Two strains of *Vibrio cholerae* non-O1 possessing somatic (O) antigen factors in common with *V. cholerae* serogroup O139 synonym 'Bengal'. Curr Microbiol 1994. In press.
19. Pearson GDN, Woods A, Chiang S, Mekalanos JJ. CTX genetic element encodes a site specific recombination system and an intestinal colonization. Proc Natl Acad Sci USA 1993; **90**: 3750–4.
20. Trucksis M, Galen JE, Michalski J, Fasano A, Kaper JB. Accessory cholera enterotoxin (Ace) the third toxin of a *Vibrio cholerae* virulence cassette. Proc Natl Acad Sci USA 1993; **90**: 5267–71.
21. Baudry B, Fasano A, Ketley J, Kaper JB. Cloning of a gene (Zot) encoding a new toxin produced by *Vibrio cholerae*. Infect Immun 1992; **60**: 428–34.
22. Miller VL, Mekalanos JJ. Synthesis of cholera toxin is positively regulated at the transcriptional level by *Tox R*. Proc Natl Acad Sci USA 1984; **81**: 3471–5.
23. Miller VL, Taylor RK, Mekalanos JJ. Cholera toxin transcriptional activator *Tox R* is a transmembrane DNA binding protein. Cell 1987; **48**: 271–9.
24. Tacket CO, Losonsky G, Nataro JP, et al. Safety and immunogenecity of live oral cholera vaccine candidate CVD 110, a △*ctx* A △*zot* △*ace* derivative of ElTor Ogawa *Vibrio cholerae*. J Infect Dis 1993; **168**: 1536–40.