CHANGING CHARACTERISTICS OF *VIBRIO CHOLERAE*: EMERGENCE OF MULTIDRUG RESISTANCE AND NON-O1, NON-O139 SEROGROUPS

MR Chandrasekhar[1], BVS Krishna[2] and Asha B Patil[3]

[1]Department of Microbiology, Raichur Institute of Medical Sciences, Raichur, India;
[2]Department of Microbiology, Royal Infirmary of Edinburgh, Edinburgh,United Kingdom;
[3]Department of Microbiology, Karnataka Institute of Medical Sciences, Hubli, India

Abstract. The serogroups and antimicrobial susceptibility patterns of *V. cholerae* isolated in Hubli, India during the years 2000 to 2004 were monitored. A total of 256 *V. cholerae* isolates were obtained during the study period, of which 129 (50.4%) belonged to serogroup O1 while the O139 and non-O1, non-O139 serogroups constituted 61 (23.8%) and 66 (25.8%) isolates, respectively. *V. cholerae* O1 Ogawa was the predominant isolate during the first 2 years of the study. However, this was replaced by *V. cholerae* non-O1, non-O139 serogroups in the following years. The *V. cholerae,* which was susceptible to most enteric antimicrobials in 2000*,* was found to be multidrug resistant in subsequent years, with the development of fluroquinolone resistance since 2002. Surveillance of the epidemiological and microbiological characteristics of *V. cholerae* provides useful information for managing cholera cases. The *V. cholerae* non-O1, non-O139 serogroups coupled with multiple antimicrobial resistance may form a group of emerging diarrheal pathogens in the tropics.

INTRODUCTION

Vibrio cholerae continues to be a challenge to public health worldwide, causing epidemics in many countries, including India. Since 1992, in addition to the existing pandemic/epidemic strain of *V. cholerae* serogroup O1, serogroup O139 has been causing disease. The spread of the *V. cholerae* serogroup O139 has involved most parts of India and its neighboring countries, where it has been responsible for large epidemics (Faruque *et al*, 2003). In many places, the two serogroups coexist causing epidemics. The *V. cholerae* non-O1, non-O139 serogroups also cause diarrheal illness resembling cholera, and have been isolated during epidemics caused by serogroups O1 and O139. Epidemiological information regarding these serogroups is limited, as most clinical laboratories generally do not look for these vibrios (Morris *et al*, 1990).

The emergence of multidrug resistant *V. cholerae* in the past several years has been a matter of concern among epidemiologists and clinicians alike. Transfer of antimicrobial resistance has been documented among various serogroups of *V. cholerae* (Rammurthy *et al*, 2000) and also between *V. cholerae* and the members of the family Enterobacteriaceae (Amaro *et al*, 1988).

As in many other regions of India, cholera epidemics occur annually in the twin cities of Hubli-Dharwad particularly during the summer months. Over the years, there has been a change in the serogroups of *V. cholerae* that has been isolated in our laboratory. Drug resistance among the *V. cholerae* isolates has also increased considerably over the past few

Correspondence: Dr Asha Patil, Department of Microbiology, KIMS, Hubli 580022, Karnataka, India. E-mail: drashapatil@rediffmail.com

years. This study analyzes the reasons and consequences of the change in serotypes and the increase in antimicrobial resistance of *V. cholerae* isolates in this region.

MATERIALS AND METHODS

The serogroups and antimicrobial susceptibility patterns of *V. cholerae* isolates from patients with acute gastroenteritis admitted to the Karnataka Institute of Medical Sciences (KIMS) hospital, Hubli and District hospital, Dharwad, India were monitored over the past 5 years from 2000 to 2004. A patient with acute gastroenteritis was defined as one who had sudden onset diarrhea within 24 to 48 hours before presentation to the hospital. A case of cholera was defined as a patient with acute gastroenteritis in whose stool sample *V. cholerae* was isolated. Patients included were those having the first episode of cholera in that year.

The number of patients suffering from acute gastroenteritis admitted to these hospitals were 384, 458, 324, 243, and 311 for the years 2000 to 2004, respectively, and included both adults and children. Stool samples were collected from all these patients and transported to the Department of Microbiology, KIMS hospital, Hubli for bacterial culture. The stool samples were processed to detect common enteric pathogens. *V. cholerae* was identified along with its biotype and serogroup by standard method (Old, 1996) Antimicrobial susceptibility testing was carried out by disc diffusion techniques using the following commercial discs (Himedia, Mumbai): ampicillin (10 µg), Co-trimoxazole (25 µg), tetracycline (30 µg), nalidixic acid (30 µg), norfloxacin (30 µg) and ciprofloxacin (5 µg) (NCCLS, 2000). The results were interpreted per published CLSI guidelines (NCCLS, 2000). Multidrug resistance was defined as resistance to three or more antimicrobials.

The strains of *V. cholerae* isolated were sent to the National Institute of Cholera and Enteric Diseases, Kolkata, India for confirmation.

RESULTS

The total number of *V. cholerae* isolated from cases of acute gastroenteritis during the 5-year period was 256. The number of *V. cholerae* isolated for the individual years from 2000 to 2004 were 93 (36.3%), 63 (24.6%), 61 (23.8%), 21 (8.2%), and 18 (7.0%), respectively. The serogroup distribution of *V. cholerae* during the study period is depicted in Fig 1. The replacement of *V. cholerae* O1 by the non-O1, non-O139 strains during the study years was statistically significant ($t = 2.8$, df = 4, 95% CI, 1.2, 74.9).

While *V. cholerae* serogroup O1 obtained during the period was 129 (50.4%), the O139 and non-O1, non-O139 serogroups constituted 61 (23.8%), and 66 (25.8%) isolates, respectively. Although *V. cholerae* O1 was the

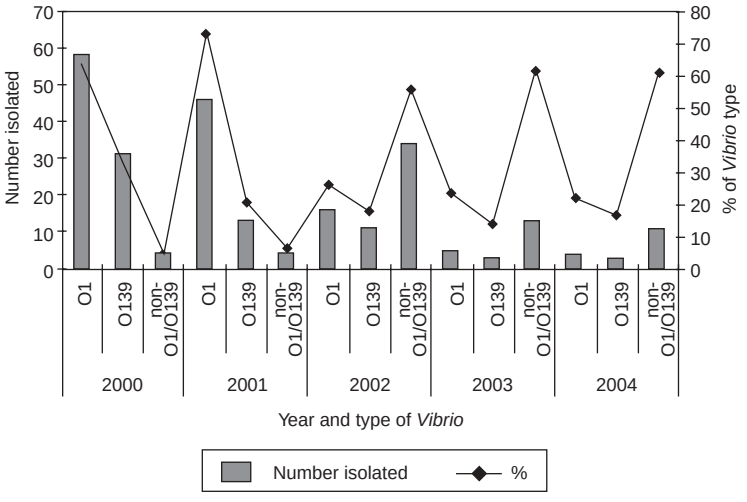

Fig 1–Numbers and percentages of *Vibrio cholerae* isolated during 2000 and 2004 in Hubli.

predominant isolate during the first 2 years, it was replaced by non-O1, non-O139 serogroups in subsequent years. *V. cholerae* non-O1, non-O139 was isolated as the sole infecting serogroup in 55 cases, while the other 11 were recovered as co-cultures from cases infected with *V. cholerae* O1.

The antimicrobial susceptibility patterns of the *V. cholerae* isolates are shown in Table 1. There were no isolates showing intermediate susceptibility to the antimicrobials tested. It is interesting to note that in the year 2000, all the isolates were susceptible to the antibmicrobials tested except for *V. cholerae* O1, some strains of which were resistant to Co-trimoxazole. In the next year, however, multi-drug resistance was noted in many isolates. The number of multi-drug resistant *V. cholerae* isolated is shown in Table 2.

From the year 2002, resistance to fluoroquinolones was observed in *V. cholerae* O1 and non-O1, non-O139 isolates, while the

Table 1
Antimicrobial susceptibility patterns of *Vibrio cholerae* isolated during 2000-2004.

Year	Total isolates	*V. cholerae* serogroup	*N* (%)	Resistance in %				
				A	Co	Na	Nx	Cf
2000	93	O1	58 (62.4)	0	22.4	0	0	0
		O139	31 (33.3)	0	0	0	0	0
		Non-O1/O139	4 (4.3)	0	0	0	0	0
2001	63	O1	46 (73.0)	60.9	80.4	87	0	0
		O139	13 (20.6)	53.9	61.5	76.9	0	0
		Non-O1/O139	4 (6.4)	50	75	75	0	0
2002	61	O1	16 (26.2)	62.5	81.3	93.8	12.5	12.5
		O139	11 (18.0)	100	27.3	100	0	0
		Non-O1/O139	34 (55.7)	82.4	61.8	94.1	55.9	47.1
2003	21	O1	5 (23.8)	80	60	80	20	40
		O139	3 (14.3)	66.7	100	66.7	0	0
		Non-O1/O139	13 (61.9)	53.9	62.5	92.3	15.4	61.5
2004	18	O1	4 (22.2)	75	75	75	50	25
		O139	3 (16.7)	33.3	66.7	66.7	0	0
		Non-O1/O139	11 (61.1)	72.7	81.8	90.9	54.6	27.3

A = amoxicillin, Co = Co-trimoxazole, Na = nalidixic acid, Nx = norfloxacin, Cf = ciprofloxacin.

Table 2
Multi-drug resistant[a] *V. cholerae* isolated in Hubli during 2000-2004.

Serogroup of *V. cholerae*	2000 *N* (%)	2001 *N* (%)	2002 *N* (%)	2003 *N* (%)	2004 *N* (%)
O1	0	16 (34.8)	10 (62.5)	3 (60.0)	2 (50.0)
O139	0	5 (16.1)	3 (27.3)	2 (66.7)	1 (33.3)
Non-O1, non-O139	0	2 (50)	17 (50)	8 (61.5)	7 (63.6)

[a]Resistant to three or more antimicrobials tested.

O139 isolates remained susceptible to these antimicrobials throughout.

DISCUSSION

Cholera epidemics continue to occur in many countries including India, with newer characteristics as the years pass by, posing challenges in its treatment and control alike. *V. cholerae* O139 which was first described to cause cholera in 1992, has spread to various countries of South Asia (Faruque *et al*, 2003), where it has co-existed with *V. cholerae* O1 strains to produce the disease. In Hubli, it was first isolated in the year 2000, along with *V. cholerae* O1 Ogawa strains (Krishna *et al*, 2002), and has continued to be isolated from acute gastroenteritis cases. The non-O1, non-O139 serogroups that initially formed a small percentage of the isolates became the predominant group isolated over the last 3 years, significantly reducing the proportion of *V. cholerae* O1 isolates. The serogrouping of non-O1 and non-O139 strains is valuable epidemiologically, but cannot be performed due to the high costs involved.

V. cholerae non-O1, and non-O139 serogroups are ubiquitous in the environment (Faruque *et al*, 2000). Data indicate the strains of *V. cholerae* that cause diarrhea have a clonal origin in the aquatic environment. *V. cholerae* strains are autochthonous to the aquatic environment. This environment serves as a reservoir of toxigenic and non-cholera toxigenic *V. cholerae* strains belonging to the O1, O139 and non-O1, non-O139 serogroups (Islam *et al*, 1990; Huq *et al*,1995). Several localized outbreaks caused by *V. cholerae* non-O1, non-O139 have been reported (Gupta *et al*, 1956; Bagachi *et al*, 1993; Dalsgaard *et al,* 1995; Sharma *et al*, 1998). Among the non-O1, non-O139 *V. cholerae* strains some serogroups (*eg*, O10, O11, O12 and O144) seem to be more often associated with disease, despite the absence of virulence factors indicating that these serogroups have a mode of pathogenesis different from that of toxigenic *V. cholerae* (Sharma *et al*, 1998). This does not appear to be a single mechanism by which these organisms cause diarrhea, and it is likely that heterogeneous factors for virulence are responsible. While some of these strains produce cholera or cholera-like toxin, the majority lack the virulence gene cassette but produce several other extracellular products, such as NAG-specific heat stable toxin, a thermostable direct hemolysin, Shiga-like toxin and hemagglutinin, play some role in the disease process (Hanne and Finkelstein, 1979; O'Brien *et al*, 1984; Honda *et al*, 1985; Bagachi *et al*, 1993).

Since the emergence of *V. cholerae* O139, new variants of the pathogen with altered genetic and phenotypic characteristics have frequently appeared (Faruque *et al*, 1997, 1999). To explain the emergence of O139 strain, two hypotheses have been proposed, that of a transposition event and a homologous recombination event replacing the O1 genes with that of O139. It has also been suggested that non-pathogenic serogroups of *V. cholerae* are the donors of the O139 specific genes (Morris, 1990). A close O-antigen relationship has been established between O139 and O 22 serogroups (Isshiki *et al*, 1996). The gene transfer event that caused the origin of the O139 serogroup may not be a unique event, but similar serotype conversions of different progenitor strains may have been occurring continually, resulting in the spread of the O139 antigen among different lineages of *V. cholerae*. Hence, the O139 antigen is present in different lineages, and this serogroup appears to comprise epidemic and non-epidemic strains derived separately from different progenitors (Faruque *et al*, 2003). Several studies also indicate continuous genetic changes in *V. cholerae* O139, leading to the emergence of yet new clones of toxigenic O139 vibrios. This process is likely to involve defined genetic

reassortment and a natural section possibly involving unidentified ecological factors and immunity of the host population (Faruque *et al*, 1997, 1999). The above observations explain the cause of the low virulence among *V. cholerae* O139 isolates during the 2000 epidemic in Hubli (Krishna *et al*, 2002).

Changes in antimicrobial resistance patterns are also likely to influence the emergence and prevalence of particular clones of *V. cholerae* (Faruque *et al*, 2003). Analysis of the *V. cholerae* strains isolated during the last 5 years revealed interesting patterns of antibiotic resistance to various common antibiotics. Although the O139 strains have remained susceptible to fluoroquinolones, all the strains of *V. cholerae* have exhibited multidrug resistance. The resistance to fluoroquinolones among the O1 and non-O1, non-O139 strains of *V. cholerae* is of particular concern. This resistance was first detected in 2002, and has increased over the years. Resistance to nalidixic acid among the O139 strains is high and this may lead to fluoroquinolone resistance due to mutations and needs close monitoring. It has been shown that non-O1, non-O139 strains play a crucial role in disseminating / mediating multidrug resistance to toxigenic O1 and O139 serogroups (Rammurthy *et al*, 2000). The rapid acquisition of drug resistance to multiple antibiotics among *V. cholerae* strains needs specific and detailed investigation as it has far-reaching and serious consequences.

Studies of cholera over the last century have provided us with a vast knowledge regarding its epidemiology, pathogenesis, and treatment. However, the mechanism resulting in the emergence of new epidemic strains and the ecology supporting such a development calls for much understanding. The constant changes in the characteristics of *V. cholerae*, either in the serogroups predominating in an outbreak, antimicrobial resistance patterns, or its virulence, need close monitoring of treatment and control of the disease. Though many clinical laboratories do not give importance to non-O1, non-O139 strains, these serogroups of *V. cholerae* can also cause clinical disease and studying their pattern provides useful information regarding evolution of the pathogen. The diversity of serogroups causing cholera may be a survival advantage to the pathogen in the wake of a less susceptible host. These strains, along with multidrug resistant clones, may form a group of emerging pathogens.

ACKNOWLEDGEMENTS

The authors are grateful to the Director of the National Institute of Cholera and Enteric Diseases, Kolkata, India, for confirming the serogrouping of the isolates.

REFERENCES

Amaro C, Aznar R, Garay E, Alcaide E. R plasmids in environmental *Vibrio cholerae* non-O1 strains. *Appl Environ Microbiol* 1988; 54: 2771-6.

Bagachi, K, Echeverria P, Arther JD, Sethabutr O, Serichantalergs O, Hoge CW. Epidemic diarrhoea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp, Thailand. *J Clin Microbiol* 1993; 31: 1315-7.

Dalsgaard A, Albert MJ, Taylor DN, *et al*. Characterization of *Vibrio cholerae* non-O1 serogroup obtained from an outbreak of diarrhoea in Lima, Peru. *J Clin Microbiol* 1995; 33: 2715-22.

Faruque SM, Ahmed KM, Siddique AK, Zaman K, Alim AR, Albert MJ. Molecular analysis of toxigenic *Vibrio cholerae* O139 Bengal strains isolated in Bangladesh between 1993 and 1996: evidence for emergence of a new clone of the Bengal vibrios. *J Clin Microbiol* 1997; 35: 2299-306.

Faruque SM, Siddique AK, Saha MN, *et al*. Molecular characterization of a new ribotype of *Vibrio cholerae* O139 Bengal associated with an outbreak of cholera in Bangladesh. *J Clin Microbiol* 1999; 37: 1313-8.

Faruque SM, Saha MN, Asadulghani, *et al*. The O139 serogroup of *Vibrio cholerae* comprises diverse clones of epidemic and nonepidemic strains derived from multiple *V. cholerae* O1 or non-O1 progenitors. *J Infect Dis* 2000; 182: 1161-8.

Faruque SM, Sack DA, Sack RB, Colwell RR, Takeda Y, Nair GB. Emergence and evolution of *Vibrio cholerae* O139. *Proc Natl Acad Sci USA* 2003; 100: 1304-9.

Gupta NP, Gupta SP, Manglik VS, Prasad BG, Yajnik BS. Investigation into the nature of *Vibrio cholerae* strains isolated from the epidemic of gastroenteritis in Kumph fair at Allahabad in 1956. *Indian J Med Sci* 1956; 10: 781-91.

Hanne HF, Finkelstein RA. Characterization and distribution of hemagglutinin produced by *Vibrio cholerae*. *Infect Immun* 1979; 36: 209-14.

Honda T, Arita M, Takeda T, Yoh M, Mieatani T. Non-O1 *Vibrio cholerae* produces two newly identified toxins related to *Vibrio parahaemolyticus* haemolysin and *Escherichia coli* heat stable enterotoxin. *Lancet* 1985; 2: 163-4.

Huq A, Colwell RR, Chowdhury MAR, *et al*. Co-existence of *Vibrio cholerae* O1 and O139 Bengal in plankton in Bangladesh. *Lancet* 1995; 345: 1245.

Isshiki Y, Kondo S, Iguchi T, Sano Y, Shimada T, Hisatsune K. An immunochemical study of serological cross-reaction between lipopolysaccharides from *Vibrio cholerae* O 22 and O139. *Microbiology* 1996; 142: 1499-504.

Islam MS, Drasar BS, Bradly DJ. Survival of toxigenic *Vibrio cholerae* O1 with common duck weed, *Lemna minor*, in artificial aquatic ecosystem. *Trans R Soc Trop Med Hyg* 1990; 84: 422-4.

Krishna BVS, Patil A, Nadgir SD, Chandrasekhar MR. Incidence of *Vibrio cholerae* serogroup O139 infection with low virulence in Hubli, Karnataka (India). *Indian J Pathol Microbiol* 2002; 46: 142-4.

Mooi FR, Bik EM. The evolution of epidemic *Vibrio cholerae* strains. *Trends Microbiol* 1997; 5: 161-5.

Morris JG Jr. Non-O group 1 *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. *Epidemiol Rev* 1990; 12: 179-91.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests. Approved standard. 7th ed. Villanova, PA: NCCLS, 2000. Document M2-A7.

O'Brien AD, Chen ME, Holmes RK, Kaper J, Levine MM. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* type 1 (Shiga)-like toxin. *Lancet* 1984; 1: 77-8.

Ramamurthy T, Rajendran K, Garg P, *et al*. Cluster-analysis & patterns of dissemination of multidrug resistance among clinical strains of *Vibrio cholerae* in Calcutta, India. *Indian J Med Res* 2000; 112: 78-85.

Sharma C, Thungapathra M, Ghosh A, *et al*. Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. *J Clin Microbiol* 1998; 36: 756-63.