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Original Article

Phage Types of *Vibrio cholerae* O1 and O139 in the Past Decade in India

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SUMMARY: Cholera has been a prevalent disease worldwide since the early 19th century. *Vibrio cholerae* O1 and O139 are the two serogroups that have been mainly implicated in causing cholera. This study reports the results of biotyping, serotyping, and phage typing of *V. cholerae* O1 and O139 (1998–2007) strains received from different parts of India for the identification of the trends in the occurrence and spread of cholera in the country. However, there has been a notable steep decline in the occurrence of *V. cholerae* O139 strains over the past few years resulting in no strain of *V. cholerae* O139 being received from any part of India in 2007 and 2008. Of the total strains received, 79.1% were serotyped as Ogawa and the remaining 20.9% were found to be Inaba, which indicates that Ogawa was the predominant serotype. Almost 100% typeability was observed with the new scheme of *V. cholerae* O1, with type 27 being the dominant phage type and *V. cholerae* O139 strains were clustered into the predominant phage type T-1. From the phage typing and serotyping results, it can be concluded that *V. cholerae* O1 (T-27) and O139 (T-1) strains circulate throughout the country at any given time.

INTRODUCTION

Cholera is a severely dehydrating, diarrheal disease, which has profoundly affected human populations, playing a significant role in their evolution and cultural development. The current pandemic, which is the 7th in the series, started in 1961 in Indonesia, reached Africa in 1971, and spread to the Americas in 1991 (1). Epidemics of cholera have been reported from different parts of India. During the past few years, severe cholera outbreaks have occurred in different parts of India, some of which were caused by antibiotic-resistant strains (2). The National Institute of Cholera and Enteric Diseases (NICED), Kolkata, regularly conducts investigations of cholera epidemics in different parts of the country. As a WHO collaborating center for diarrheal disease research and training, NICED is a reference laboratory and receives approximately 1,000 to 1,500 strains of *Vibrio cholerae* per year from different parts of India and other countries for biotyping, serotyping, and phage typing.

V. cholerae is classified into different serogroups based on their lipopolysaccharide composition, of which only the serogroups O1 and O139 are known cause cholera. The O1 serogroup is further classified into two biotypes, classical and El Tor and each biotype into two major serotypes Ogawa and Inaba (3). Before 1961, most epidemics were caused by the classical biotype; however, the El Tor biotype is responsible for the ongoing 7th pandemic. In the year 1993, *V. cholerae* O139 came into the limelight by causing explosive cholera epidemics in the Indian subcontinent (4).

The use of phage typing as a method of classifying *V. cholerae* strains has contributed greatly to the understanding of the epidemiology of cholera, particularly before the advent of the molecular typing techniques (5). A phage-typing scheme introduced by Basu and Mukerjee for *V. cholerae* O1 biotype El Tor was efficiently used to characterize the El Tor biotype of *V. cholerae* O1 (6,7). However the limitations and restrictions of this scheme prompted us to develop a new phage-typing scheme for *V. cholerae* O1 biotype El Tor (8,9). In 1992, yet another serogroup, namely O139, caused outbreaks of cholera in geographically distinct areas in India (4). This novel strain was first isolated in the southern part of India and characterized and confirmed at NICED (4). The sudden appearance of the O139 serogroup in late 1992, its rapid spread throughout Southeast Asia in 1993 followed by a quiescent period during 1994–1995, and its subsequent emergence in 1996 and 1997 are inadequately understood, but epitomize the unpredictable nature of cholera epidemiology. Within a few months of the first appearance of O139, it spread to other cholera endemic areas in India (10). The reemergence of O139 in 1996 indicates that its appearance was not a one-time event and that the serogroup has the potential to persist and spread to other continents (10,11). The occurrence of epidemics caused by *V. cholerae* O139 is a significant turning point in the history of cholera because the evidence points to this strain arising (11) from genetic recombination and horizontal gene transfer, and the acquisition of unique DNA. Subsequently, another phage-typing scheme was developed for the newly emerged *V. cholerae* O139 in 2000 (12). These two phage-typing schemes specific for *V. cholerae* O1 and O139 are being routinely used for the classification of strains of *V. cholerae* O1 and O139

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This study reports the results of biotyping, serotyping, and phage typing of *V. cholerae* O1 and O139 (1998–2007) strains received from different parts of the country by NICED in the past decade and attempts to identify trends in the occurrence and spread of cholera in the country.

MATERIALS AND METHODS

Strains: From 1998 to 2007, a total of 7,473 strains of *V. cholerae* O1 and 1,057 strains of *V. cholerae* O139 from different parts of India were received at NICED for biotyping, serotyping, and phage typing. Of the 7,473 strains of *V. cholerae* O1 that were received, 5,374 were included in the present study.

Bacteriology: Isolates were confirmed to be *V. cholerae* O1 and O139 using the standard techniques (13). Serological identification was performed using polyvalent O1, O139, and subsequently monospecific Inaba and Ogawa antisera (Difco, Detroit, Mich., USA).

Phage-typing procedure: Phage typing was performed based on the standard methodology routinely performed in our laboratory (8,14,15). A single colony of *V. cholerae* strain from nutrient agar was inoculated into nutrient broth (5 ml) and incubated under stationary conditions for 4–5 h at 37°C. An aliquot of the young broth culture (0.1 ml) was mixed with molten 0.8% soft agar (3.5 ml, maintained at 42°C) and poured onto a nutrient agar plate. The aim was to produce a uniform lawn of *V. cholerae* O1 and O139 grown on the surface of the agar, thereby providing an adequate substrate for phage action, but not so heavy as to obscure the plaque. The plates were then allowed to dry at room temperature (20–30 min), followed by the addition of 0.05 ml of phage lysate (1×10^4 pfu/ml) from the routine test dilutions (RTD) (8). The plates were maintained at room temperature for 10 min to allow the drop to dry and then incubated at 37°C for 16–18 h. *V. cholerae* MAK 757 (ATCC 51352) was used for each set of experiment as a control, since this strain is lysed by the O1 group of phages. Similarly, NPR-4 (14) was used as the other control, since this is lysed by the O139 group of phages. After incubation, each reaction was recorded as positive if five or more plaques were formed.

RESULTS AND DISCUSSION

We have previously reported the distribution of biotypes, serotypes, and phage typing of *V. cholerae* O1 strains during the period 1991–1998 (9). As shown in the Fig. 1, the *V. cholerae* strains were received from different geographical regions of India, excluding the states of Jammu and Kashmir, Himachal Pradesh, and Arunachal Pradesh, from where no strains of *V. cholerae* O1 or O139 were received. However, new areas that had not witnessed the disease before were added to the cholera map of India (Figs. 1 and 2). The new states that were added to the cholera map were the Andaman & Nicobar Islands, Kerala, Sikkim, and Tripura from where *V. cholerae* isolates were reported. NICED also received strains of *V. cholerae* from a couple of new medical research institutions from different parts of the country.

All strains received in this study were characterized and confirmed to be *V. cholerae* O1 biotype El Tor. A new variant of the El Tor biotype of *V. cholerae* O1, which produces cholera toxin of the classical biotype, has recently been reported from different parts of Asia and Africa (16,17). Chronological studies in Kolkata have shown that the new variant of the El Tor biotype came into existence between 1994 and 1995 and that all the strains of the El Tor biotype currently isolated in Kolkata belong to this category (18). In this study, we did not determine the type of cholera toxin possessed by the El Tor biotype strains received from different parts of India; this characterization is currently in progress.

Of the total number of strains received, 96.5% were serotyped as Ogawa and the remaining 3.5% were Inaba. In 1998 and 1999, Ogawa was the only serotype reported, whereas the Inaba serotype was not received from any part of the country. However, from 2000 to 2007, both Ogawa and Inaba serotypes were encountered, although Ogawa was the predominant serotype.

State	City Number*	City Name	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Andhra Pradesh	1	Vishakhapatnam		21	31	13	2	4				
Andaman & Nicobar Island	2	Port Blair								16		
Assam	3	Guahati								18	17	19
Bihar	4	Patna						1				
Delhi	5	Delhi	87	75	94	40	58			183	111	83
Goa	6	Panaji		15	3	1	4	3	22			
Gujrat	7	Baroda	47	115	60	78	63	25	41		97	87
	8	Surat	23	155	40	36	52	28	52		87	67
Karnataka	9	Bangalore	3	15	17	2	3	2	2			
Kerala	10	Cochin							2	12		
Madhya Pradesh	11	Bhopal			11	3	4					
	12	Indore						10		15		
Maharashtra	13	Aurangabad	9	149	31	50	62	11	22		121	88
	14	Kolhapur		12	3	1	1			34	12	11
	15	Mumbai	75				40	24	79	197	79	98
	16	Nagpur	77	152	64	24	34	37	14	173	113	93
	17	Pune	50	35	36	16	12	68	45	187	97	81
	18	Sholapur	2	15	16	2	2			11	14	13
Orissa	19	Bhubaneswar		19	2	2	1	27	15	13	7	
Punjab	20	Chandigarh					57	62		111	21	77
	21	Ludhiana	62	150	21	143	46		140			91
Rajasthan	22	Bikaner	20	5					12	17	17	
Sikkim	23	Gangtok							1			
Tamilnadu	24	Chennai	91	158	58	66	57	83	42	191	101	97
	25	Madurai					2	65	62			24
	26	Pondicherry		9	4	1	2	14	1	23	23	
Tripura	27	Agartala									7	
Uttar Pradesh	28	Kanpur									3	5
	29	Lucknow	4									
West Bengal	30	Kolkata					9	38	70	98	54	

*This number has been mentioned in the map. We received strains in these years.

Fig. 1. Map of India showing the areas from where *V. cholerae* O1 biotype El Tor strains were received during the period 1998 to 2007.

State	City Number*	City Name	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Delhi	1	Delhi			20					7	8	
Gujrat	2	Ahmedabad	36		40			1				
Karnataka	3	Bangalore	33		40		1					
Maharashtra	4	Mumbai	215		140	3	45	8	5	2		
Punjab	5	Chandigarh					2					
TamilNadu	6	Chennai	170		110			26	16			
West Bengal	7	Kolkata	129									

*This number has been mentioned in the map. We received strains in these years.

Fig. 2. Map of India showing the areas from where *V. cholerae* O139 biotype El Tor strains were received during the period 1998 to 2007.

Table 1. Distribution of phage types of *V. cholerae* O1 biotype El Tor strains in India during the period 1998 to 2007

Year	Strains received	Strains analyzed	Serotype		Basu & Mukerjee			New phage type[1)]					
			Ogawa (%)	Inaba (%)	T_2 (%)	T_4 (%)	UT	T-27 (%)	T-26 (%)	T-25 (%)	T-19 (%)	T-13 (%)	T-7 (%)
1998	550	403	100	0	98.5	1.5	0	74.2	5.5	0	4.5	3.7	2.5
1999	1,100	732	100	0	34.7	53.4	11.9	63.8	8.6	2.2	2.6	0	0
2000	491	357	98.6	1.4	7.8	85.2	7.0	68.6	14.0	2.8	5.8	0	0
2001	478	314	99.7	0.3	87.3	0	12.7	87.3	3.8	0	1.2	0	2.2
2002	511	468	100	0	84.2	0	15.8	73.9	3.9	3	5.1	0	2.1
2003	502	398	99.0	1.0	1.8	91.8	6.4	83.2	4.1	4.9	2.3	3.1	2.4
2004	622	419	95.5	4.5	1.2	90.6	8.2	78.1	8.0	1.1	5.4	0	5.6
2005	1,304	1,005	97.2	2.8	61.5	30.2	8.3	80.5	5.8	2.1	2.4	3.8	0
2006	981	678	88.5	11.5	60.8	28.2	11.0	78.2	3.7	4.9	4.6	5.1	0
2007	934	600	91.1	8.9	80.3	2.0	17.7	83.0	1.3	0.8	0.7	0.5	0.3
Total	7,473	5,374	96.5	3.5	53.4	36.3	10.2	76.8	5.7	2.3	3.3	1.8	1.1

[1)]: Table 1 includes only the major types (phage types with highest number of strains) of new phage typing scheme of each year.

Over the past few years, Ogawa has been observed to be the dominant serotype by several researchers (19,20). Periodic shifts in the occurrence of Ogawa and Inaba serotypes in a given area is a usual phenomenon and is thought to be a consequence of population-level immunity patterns (21). What was of interest in the present study, was the finding that the prevalence of a serotype was not spatially restricted but seemed to occur across the country. This would indicate that within the country one serotype prevailed. The cyclical serotype dynamics seen in cholera may be the result of high, but incomplete, cross-immunity between cholera serotypes (21).

All of the 5,374 strains clustered into either type 2 or type 4 phage types when phage were typed using Basu and Mukerjee's scheme (Table 1). There was a shifting trend in the distribution of the strains between these two types, with all strains received in 2001 and 2002 belonging to phage type 2. Again, in 2003 and 2004, almost all typed strains (98%) belonged to type 4, although 10–20% remained untypable. However, all these strains were phage typed with a new phage-typing scheme. Almost 100% typeability was observed using the new scheme, with type 27 being the dominant phage type. In this study, the strains belonging to type 27 showed variability with Basu and Mukerjee's scheme. On the basis of this variation we can divide these strains in to three groups, T2-T27 (52.5%), T4-T27 (32.8%), and UT-T27 (14.7%), suggesting that three closely related clones were disseminated in India. The common types other than type 27 in the order of frequency, were types 26 (5.7%), 25 (2.3%), 19 (3.3%), 13 (1.8%), and 7 (1.1%). The distribution of phage types did not follow any regular pattern and appeared to be scattered in different parts of the country, as seen previously (9). Our study shows that type 27 was the dominant type in all parts of the country, followed by type 26. From the phage typing and serotyping results, it is evident that a clonal complex of the *V. cholerae* O1 (T 27) strain circulates throughout country at any given time.

In contrast to the wide distribution of *V. cholerae* O1, during the study period all of the 1,057 *V. cholerae* O139 strains were collected from only 7 states (Fig. 2). In the year 1998, the strains were received from 5 states (n = 583) but reports of *V. cholerae* O139 in India were

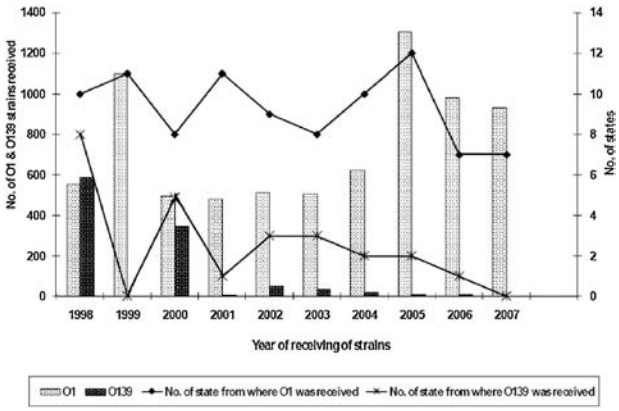

Fig. 3. Distribution of *V. cholerae* O1 and O139 strains received during the period 1998 to 2007.

absent in 1999. Following reemergence (n = 350) in 5 states in 2000 (Fig. 2), over the past few years beginning from 2001 onward, *V. cholerae* O139 infection has been observed to be restricted to the areas of 1 to 3 states. Furthermore, there was a gradual decline in the number of *V. cholerae* O139 strains throughout the country (Figs. 2 and 3). No strains of *V. cholerae* O139 were received from any part of India in 2007. Of the total 1,057 *V. cholerae* O139 strains examined, 62.9% belonged to the predominant phage type T-1, while 37.1% belonged to a further 9 phage types. Phage typing of *V. cholerae* O139 isolated in 1998 revealed a mixture of all the phage types. However, when reemerging in 2000, the predominant type was found to be T-1 along with two other minor types, T-8 and T-10, which were isolated in the year 1998, implying that there was a population bottleneck and selective sweeping had occurred.

V. cholerae appeared abruptly in late 1992. Thereafter it disappeared, but reemerged in 1996. The reemergence of O139 in 1996 indicated that its appearance was not a chance event and that the serogroup has the potential to persist and spread to other continents (10). The cyclical reemergence of *V. cholerae* O139 observed in the present decade, and a pathogen-based monitoring system, will play an important role in aiding an understanding of the biological aspect of this pathogen along with its effect on public health.

Overall, *V. cholerae* O1 continues to rank as the major cause of cholera in India. This trend was also evident in bacteriological and epidemiological investigations carried out in the cholera-endemic areas of Kolkata which revealed the complete dominance of *V. cholerae* O1 in hospitalized cases of acute diarrhea admitted to the infectious diseases hospital in Kolkata (22,23). In the light of the availability of a variety of molecular typing techniques, the usefulness of phage typing has diminished over the years. We, however, continue to use phage typing as it allows us to compare the phage types of *V. cholerae* O1 with those isolated several decades ago when phage typing was initiated in this institute. At a national level, it allows us to observe any sudden changes in the phage types across the country.

Conflict of interest None to declare.

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