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Phenotypic and antibiogram pattern of *V. cholerae* isolates from a tertiary care hospital in Mumbai during 2004–2013: a retrospective cross-sectional study

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ABSTRACT

Objectives: Cholera is a major gastroenteric disease with reports on fluctuation and resistance. Hence, the objective is to determine the trend in seasonality, resistance pattern, prevalent biotypes, serotypes and phage types between 2004 and 2013 among *Vibrio cholerae* isolates.

Design: A retrospective cross-sectional study.

Settings: A single-centre study was carried out at a tertiary care hospital in a metropolitan city (Mumbai) of a developing country (India).

Methods: Records of stool specimen cultures of patients with suspected cholera from January 2004 to December 2013 were analysed. The organisms were identified as per standard protocol. Antimicrobial susceptibility testing was performed as per Clinical Laboratory Standard Institute. Biotyping, serotyping and phage typing were carried out. From the confirmed cases of cholera, demographic and laboratory details were noted. Descriptive analysis was used and the data were presented in the form of percentages.

Results: *Vibrio cholerae* was predominant in males and was isolated from 9.41% (439/4664) of stool specimens. Variability was found in terms of the gross appearance of stool specimens, seasonal trend and antibiotic resistance pattern. The antimicrobial susceptibility showed a waxing and waning pattern for most of the antibiotics (ampicillin, cefuroxime, chloramphenicol, tetracycline) tested, while for a few others the strains were either uniformly sensitive (gentamicin, norfloxacin) or resistant (trimethoprim-sulfamethoxazole, nalidixic acid). All isolates belonged to subgroup O1 and biotype El Tor. The most common serotype was Ogawa. The predominant phage type was T2 (old scheme) and T27 (new scheme).

Conclusions: The predominant biotype, serotype and phage type were El Tor, Ogawa and T27 phage, respectively. The changing trends in antimicrobial resistance pattern over the years necessitate continued epidemiological and microbiological surveillance of the disease.

Strengths and limitations of this study

- Dissemination of such data is important for public health practices.
- Performance of biotyping, serotyping, antibiogram and phage typing.
- The data showed the changing trends over the years and the rising antimicrobial resistance which necessitate a continued surveillance study of cholera disease in Mumbai.
- This manuscript was prepared based only on the phenotypic pattern of *Vibrio cholerae*.
- The genotypic pattern and comparison between the two could not be carried out.

INTRODUCTION

Cholera is a major public health threat affecting the poor in developing countries[1] and continues to spread across many countries. Historical records suggest that cholera-like disease may have been first reported in the Era of Hippocrates (460–377 BC).[2] In the year 2014, a total of 190 549 cases of cholera were reported from 42 countries with a case fatality rate of 1.17%.[1] During the same year in India, a total of 4031 cholera cases were reported from 12 different states, with 21 deaths.[1] According to the International Health Regulations (2005), notification of all cases of cholera is no longer mandatory and this leads to under-reporting, as observed by the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India.[1 3] The different causes of under-reporting could be a fear of negative impact on travel and trade in affected areas, limited surveillance of the disease, inconsistent case definitions and deficiency of laboratory diagnostic services.[4 5] Integrated Disease Surveillance Project in

India is a state-based surveillance system which reports cholera.[6] However, reporting of cholera is still inadequate in India.[7]

The disease occurs throughout the year, the majority reported during the monsoon season. The causative agent, *Vibrio cholerae (V. cholerae)*, has two biotypes, classical and El Tor.[8] Each biotype has three serotypes: Ogawa, Inaba and Hikojima.[9 10] *V. cholerae* have demonstrated changes in prevalent biotypes and resistance over time.[11 12] The El Tor biotype (seventh pandemic) replaced the classical biotype causing the first six pandemics of cholera. Phage typing has contributed greatly to the understanding of the epidemiology of cholera. Phage typing at the NICED, Kolkata, India included the Basu and Mukherjee[13] typing and new phage typing schemes.[14]

The present study was carried out at a 2200 bedded tertiary care teaching hospital in the metropolitan city of Mumbai. As per the 2011 census, the population of Mumbai city was 124 million.[15] Mumbai city is located on the western coast of Maharashtra along the banks of the Arabian sea and falls at latitude and longitude of 18° 55′ N and 72°54′ E, respectively. In Mumbai, 614 cases per thousand population are annually reported diarrhoeal disease burden.[16] The microbiology department of our institute receives an average of 500 stool specimens annually from diarrhoeal cases for bacteriological study. The fluctuations in disease numbers and changing trends in resistance incited this study with an objective to determine trends in the seasonal association, resistance pattern, prevalent biotypes, serotypes and phage types of *V. cholerae* isolates over a period of 10 years.

METHODS

Procedure

A retrospective analysis of microbiology records of stool culture from January 2004 to December 2013 was carried out and the data were analysed on a yearly basis. Stool specimens were processed directly and after enrichment in alkaline peptone water as per standard protocol.[17] The quality control of media and antibiotic discs used were carried out as per standard protocol. Specimens were inoculated on 5% sheep blood agar, MacConkey's agar and Thiosulphate citrate bromothymol blue sucrose agar (HiMedia Laboratories Pvt, Mumbai). Specimens were also plated after enrichment in alkaline peptone water. Plates were incubated aerobically at 37°C and read after 24–48 hours. Organisms were identified as per standard microbiological procedures. Non-lactose fermenting isolates which were Gram-negative motile bacilli, oxidase and catalase positive, fermenting glucose, sucrose and lactose (late) without gas, nitrate reducers, indole positive and citrate negative, lysine positive, arginine negative, ornithine positive, Cholera Red reaction positive and string test positive were presumptively identified as *V. cholerae*. The isolate identification was confirmed using *V. cholerae* polyvalent, Ogawa and Inaba antisera supplied by Becton Dickinson (Difco *V.cholerae* antiserum poly; Difco *V.cholerae* antiserum Ogawa; Difco *V. cholerae* antiserum Inaba). Biotyping was carried out as per standard phenotypic tests. Clinical and laboratory details of patients such as age, gender, residential address, date of specimen receipt, gross appearance of stool, reports of the hanging drop, culture and antibiotic sensitivity were noted. Repeat isolates from the same patient were excluded. Antimicrobial susceptibility test was carried out by the Kirby-Bauer disc diffusion method as per Clinical Laboratory Standard Institute.[18] ATCC strain *Escherichia coli* 25922 was used for quality control of antimicrobial sensitivity testing.[18] The following antibiotic discs (HiMedia Pvt, Mumbai) were tested—tetracycline (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75), chloramphenicol (30 µg), cefuroxime (30 µg), ampicillin (10 µg), gentamicin (10 µg), furazolidone (100 µg), norfloxacin (10 µg) and nalidixic acid (30 µg). The isolates were preserved in Brain Heart infusion agar with 1% sodium chloride.[19] The preserved isolates were overlaid with sterile paraffin oil before transporting to NICED, Kolkata. Phage typing was carried out on 214 isolates by NICED, Kolkata from 2005 onwards using conventional the Basu and Mukherjee[13] phage typing scheme and the New phage typing scheme.[14] For the purpose of this study, a multidrug resistant (MDR) organism was defined as resistance to two or more classes of antimicrobial agents.

Statistical analysis

Descriptive analysis was used and presented in terms of percentage. The parameters analysed included age and gender distribution, residential address, month and year, gross findings of stool, results of the hanging drop, biotype, serotype, phage type, resistance pattern of the isolate and their changing trends.

RESULTS

Demographics of the study participants

About 4664 stool specimens of suspected cases were processed by microbiological culture during the study period. *V. cholerae* was isolated in 439 patients (9.41%). Of the 439 patients, 313 (71.3%) were adults and 126 (28.7%) were children. The male to female ratio in adults was 2.6:1. In paediatric patients, the male to female ratio was 1.1:1. Considering the place of residence of patients infected with *V. cholerae* (n=264), the majority (71.2%) resided within a 5 km radius from the institution with no particular predominance of any area.

Trend of *V. cholerae* cases and characteristics of stool specimens

The cases were detected throughout the year, but peaked in the month of July for all the years and tapered from September onwards (table 1). A maximum number of cases (n=79) were recorded in the year 2004,

Table 1 Year-wise distribution of *V. cholerae* isolation

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2004	0	0	3	5	15	15	14	19	3	1	4	0	79
2005	0	1	7	2	0	2	10	23	2	1	0	1	49
2006	0	0	0	3	1	4	8	1	6	6	1	0	30
2007	0	0	5	10	10	4	40	NC	NC	NC	NC	NC	69
2008	NC	NC	NC	NC	NC	2	7	10	11	7	1	5	43
2009	5	8	0	0	1	2	30	9	12	2	2	0	71
2010	0	3	5	0	6	0	14	13	2	2	3	4	52
2011	1	4	0	5	2	0	1	4	3	1	1	0	22
2012	0	0	0	0	1	1	1	0	1	0	0	0	4
2013	0	1	0	0	0	1	11	4	3	0	0	0	20
Total	6	17	20	25	36	31	136	83	43	20	12	10	439

NC, culture of stool specimens not performed; *V. cholerae, Vibrio cholerae.*

Table 2 Year-wise distribution of different serotypes of *V. cholerae*

	Serotypes		
Year	Ogawa	Inaba	Total
2004	79	0	79
2005	47	2	49
2006	11	19	30
2007	69	0	69
2008	40	3	43
2009	68	3	71
2010	47	5	52
2011	22	0	22
2012	4	0	4
2013	20	0	20
Total	407	32	439

V. cholerae, Vibrio cholerae.

followed by 2009 (n=71). Records of gross appearance were available in 368 stool specimens. Of this 77.4% (285) were rice watery. The remaining were green liquid 10% (37), yellow liquid 8.2% (30) or brown liquid specimens 3.8% (14). *V. cholerae* was also isolated from one specimen of green semisolid and reddish liquid stool.

Biotypes and serotypes of *V. cholerae* isolates

Of the 439 isolates identified as *V. cholerae*, data for hanging drop were available from 327 specimens which were culture positive. Hanging drop was positive in 60.2% (n=197) of culture-positive specimens. All isolates belonged to subgroup O1, biotype El Tor. The most common serotype was Ogawa (407;92.7%). No Inaba was isolated in 2004, 2007, 2011, 2012 and 2013 (table 2).

Antimicrobial sensitivity pattern of the isolates

More than 85% of the isolates were found to be sensitive to gentamicin and more than 90% to norfloxacin (table 3). On the other hand, <7% were sensitive to nalidixic acid and <27% to trimethoprim-sulfamethoxazole throughout the study period. For the other antimicrobials, varying trends were observed. The strains were maximally sensitive to tetracycline throughout the years except in 2008 (51.2%). The sensitivity of *V. cholerae* to ampicillin reduced from 67.1% in 2004 to 46.2% in 2010, and subsequently all strains were ampicillin resistant. Sensitivity to chloramphenicol increased from 0% in 2004 to 100% in 2007, 2008 and 2009. It reduced to 20% in 2013. Susceptibility to cefuroxime increased from 34.2% in 2004 to 100% in 2008 and then decreased to 25% in 2012. Furazolidone sensitivity varied from 7.6% (2004) to 100% (2012). No interserotype difference in resistance pattern was noted. Throughout the decade, the treatment for cholera in our institute was doxycycline.

An analysis was also carried out to determine the changing trend in MDR (figure 1). Resistance to three or more than three antimicrobials tested was 96.2% in 2004, which decreased to 51.9% in 2010. However, in 2013, this increased to 90%. Resistance to any two antimicrobials increased from 2.5% in 2004 to 43.7% in 2009 and then decreased to 10% in 2013. Throughout the study period, only one isolate was susceptible to all the antimicrobials tested (2004).

Phage types of *V. cholerae* isolates

A total of 214 strains of *V. cholerae* were sent for phage typing. The results of phage typing revealed Basu and Mukherjee type T2 to be the predominant phage (84.6%). Non-typable strains (NT) accounted for 13.08% and Untypable strains (UT) accounted for 2.3%. The New phage typing scheme showed T27 as the predominant phage (77.6%), followed by T14 (1.9%), T23 and T26 (1.4%), T24 (0.9%), T13, T20 and T25 (0.46%). NT accounted for 13.08% and UT accounted for 2.3%. No trend in phage type was observed.

DISCUSSION

In this study, *V. cholerae* was isolated from 9.41% of suspected cases, maximal during the monsoon season with El Tor, Ogawa and T27 being the most prevalent biotype, serotype and phage type, respectively. About one-third of cases (28.7%) were reported of children. *V.*

Table 3 Antimicrobial susceptibility patterns of *V. cholerae* isolates during 2004–2013 (% susceptible)

Year of isolation (n)	TE n (%)	AM n (%)	CH n (%)	GM n (%)	NF n (%)	CO n (%)	FZ n (%)	NA n (%)	CE n (%)
2004 (79)	77 (97.5)	53 (67.1)	1 (0.01)	78 (98.7)	75 (94.9)	7 (8.9)	6 (7.6)	3 (3.8)	27 (34.2)
2005 (49)	42 (85.7)	19 (38.8)	0 (0)	46 (93.9)	45 (91.8)	1 (2)	4 (8.2)	1 (2)	26 (53.1)
2006 (30)	27 (90)	15 (50)	15 (50)	30 (100)	27 (90)	4 (13.3)	3 (10)	2 (6.7)	24 (80)
2007 (69)	68 (98.6)	67 (97.1)	69 (100)	67 (97.1)	68 (98.6)	9 (13)	12 (17.4)	4 (5.8)	68 (98.6)
2008 (43)	22 (51.2)	34 (79.1)	43 (100)	42 (97.7)	42 (97.7)	6 (14)	7 (16.3)	0 (0)	43 (100)
2009 (71)	59 (83)	25 (35.2)	71 (100)	68 (95.8)	69 (97.2)	19 (26.8)	18 (25.4)	5 (7)	68 (95.8)
2010 (52)	44 (84.6)	24 (46.2)	51 (98.1)	52 (100)	49 (94.2)	14 (26.9)	44 (84.6)	3 (5.8)	38 (73.1)
2011 (22)	22 (100)	0 (0)	20 (90.9)	22 (100)	22 (100)	5 (22.8)	8 (36.4)	0 (0)	22 (100)
2012 (4)	4 (100)	0 (0)	0 (0)	4 (100)	4 (100)	0 (0)	4 (100)	0 (0)	1 (25)
2013 (20)	19 (95)	0 (0)	4 (20)	17 (85)	18 (90)	2 (10)	3 (15)	0 (0)	5 (25)

AM, ampicillin; CE, cefuroxime; CH, chloramphenicol; CO, trimethoprim-sulfamethoxazole; FZ, furazolidone; GM, gentamicin; NA, nalidixic acid; NF, norfloxacin; TE, tetracycline; *V. cholerae, Vibrio cholerae*.

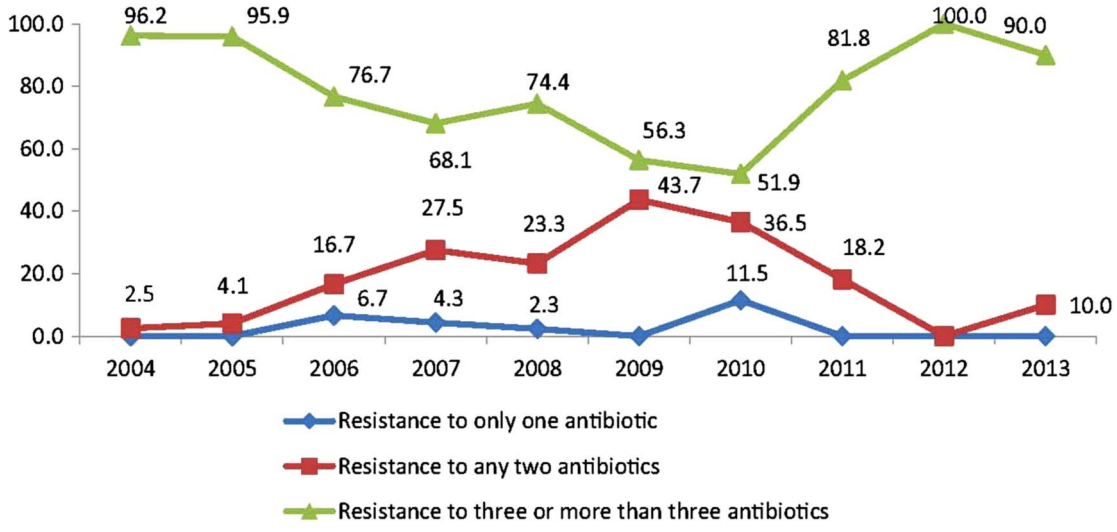

Figure 1 Changing trend in multidrug resistance pattern of *V. cholerae* (10 years). *V. cholerae, Vibrio cholerae.*

cholerae infection was common in males compared to females in the adult and paediatric population.[20 21]

A rising resistance was observed to most of the antimicrobials tested.

The isolation rate of *V. cholerae* from suspected cases was 9.41%, which is similar to other studies.[20 22 23] Previous studies from Mumbai reported culture positivity rates ranging from 7.2% to 20%.[23 24] Interestingly, *V. cholerae* was also isolated from stool specimens which were other than rice-watery. This may be due to the sample being collected during the initial infection period or due to mixed infection. Hence, irrespective of colour and consistency, the stool specimen should be cultured and investigated for *V. cholerae*.

The initial investigation on the stool specimen was hanging drop preparation. Our study showed that only 60.2% of culture positive specimens were positive for hanging drop. The reason for the high negativity rate may probably be attributed to the difficulty in differentiating between darting motility and actively motile organisms, delay in transport of specimens to the laboratory and effect of antibiotic therapy. Kulkarni *et al*[25] have reported hanging drop positivity in 74% of culture-positive specimens.

A seasonal trend in the isolation of *V. cholerae* was seen. Cases initiated in February, peaked in July and tapered after September. This closely mimics the seasonal trends in monsoon. As per the data available from the India water portal organisation, the rainfall in Mumbai begins in June, peaks in July and gradually decreases in the month of August and by October it is negligible.[26] The monsoon-related nature of disease coupled with the ability of *V. cholerae* to grow rapidly in warm humid environment and with Mumbai city receiving heavy showers with frequent flooding during monsoon, could be attributed to this seasonal trend of the disease.[22 23]

Throughout the years, resistance to tetracycline was low (<20%) except in 2008 (49%). High-level tetracycline resistance was noticed by Kar *et al*[27] from Odisha (2010), Bhattacharya *et al*[28] from Kolkata (2007–2009), Kumar *et al*[29] from Rayagada, Odisha (September–October 2010), Borkakoty *et al*[30] from Assam (2007,

2008 and 2010) and Taneja *et al*[31] from Chandigarh (2008). Plasmids are responsible for tetracycline resistance in *V. cholerae*. Vibrios do not stably carry plasmids, and hence the resistance pattern fluctuates.[31] It is also possible that the irrational use of tetracycline or doxycycline in previous years could have led to the rapid emergence and spread of tetracycline-resistant isolates.[31] Doxycycline is the drug of choice for the treatment of cholera in this institute and in the city. This is consistent with the susceptibility pattern over the years. Observing its continued therapeutic efficacy, it is recommended that the drug should be used judiciously for the management of acute diarrhoeal diseases in order to delay the development of resistance to the drug.

There was an increase in resistance to ampicillin in 2005 (61%) with total resistance being observed during 2011–2013. Similar high rates of ampicillin resistance have been reported by Sharma *et al*[32] from Delhi (2003 to 2005) and Palewar *et al*[20] from Pune (2010 to 2012). One hundred per cent ampicillin resistance was also reported by Pal *et al*[33] from the Eastern coast of Odisha (April–July 2009) and Kar *et al*[27] from Odisha (2010).

Resistance to chloramphenicol was seen in spurts during 2004–2006, 2012 and 2013. For other years, sensitivity was more than 90%. Goel and Jiang[34] during 2004–2007, Pal *et al*[35] during 2007 and Palewar *et al*[20] during 2010–2012 reported 100% sensitivity to chloramphenicol. Kar *et al*[27] reported 100% resistance to chloramphenicol during 2010.Trimethoprim-sulfamethoxazole, furazolidone and nalidixic acid showed high levels of resistance similar to other studies.[20 27 32–37] Trimethoprim-sulfamethoxazole is the drug of choice in younger children. In view of the high level of trimethoprim-sulfamethoxazole resistance and contraindication for use of tetracycline and fluoroquinolones in children, other options need to be considered. Low level of resistance was observed to norfloxacin (<10%) and gentamicin (<15%) throughout the study period. This finding is similar to other studies.[20 27 32 35 36] A rising trend in MDR was observed over the years. A similar finding has been reported globally.[37 38] The probable cause could be either due to spontaneous mutations or horizontal transfer of resistant genes within the gut flora and Vibrio species.[37] The changing trend in antibiotic resistance over time may indicate the variability of treatment in the community.

The El Tor biotype of *V. cholerae* has completely replaced the classical biotype. The results of this study confirm these reports.[38] Studies from India report the emergence of *V. cholerae* O139.[39–42] However, in this study, all isolates were *V. cholerae* serogroup O1. The predominant serotype was Ogawa (93.26%) with an occasional isolation of Inaba (6.74%). In 2006, Inaba accounted for 63.3% of all isolates. This trend was similar to other studies.[24 36] A periodic shift from Ogawa to Inaba has been reported either due to mutational changes in gene coding for serotype specificity[43] or as a result of selection due to immune response during cholera infection[44] or due to genetic reversal.[45]

The predominant phage type by the Basu and Mukherjee phage typing scheme was T2 (84.6%). The new typing scheme showed T27 as the predominant phage type, which is similar to other studies.[20 21 24 25 32 36]

CONCLUSION

El Tor Vibrios continue to be the predominant biotype, Ogawa the serotype and T27 the phage type. The changing trends over the years and the rising antimicrobial resistance necessitate continued epidemiological and microbiological surveillance for the disease.

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