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A Bacteriological Study of *Vibrio cholerae* Isolated from Rural Tertiary Care Hospital of Loni, Western Maharashtra

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Authors' contributions

Author NAR performed lab work, author SS guided and helped in directing the work. Authors NAR, SS contributed in study design. Authors DB and SD contributed in data collection and analysis. SB did phage typing of isolates. Authors NAR, SS, DB and SD all interpreted data and contributed in write up. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: Cholera is endemic in many parts of India and a major public health problem. The present study was carried out with the aims to understand biotype, serotype, phage type and drug resistance of *Vibrio cholerae* isolates obtained at a rural tertiary care hospital in Loni.
Study Design: Descriptive retrospective study was carried out to study *V. cholerae* isolates from 544 faecal specimens of patients with acute gastroenteritis.
Place and Duration of Study: The study was conducted during 2009-2012 at Rural Medical College of Pravara Institute of Medical Sciences, Loni, District Ahmednagar, Maharashtra, India.
Methods: A total of 28 isolates of *V. cholerae* were included in the study. *V. cholerae* was identified by standard microbiological procedures. Biotyping, serotyping and phage typing was done. Antibiotic sensitivity testing was performed by Kirby- Bauer disc diffusion method.
Results: *V. cholerae* strains were isolated from 28 faecal specimens. All the isolates were identified as *V. cholerae* O1 biotype El Tor serotype Ogawa and phage 27 was the

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predominant type. Male: Female ratio was 1:1.5 and high incidence was seen in 0-10 age group (35.71%). Maximal occurrence in monsoon season was recorded. All the isolates were resistant to co-trimoxazole, nalidixic acid and ampicillin. However maximum sensitivity was observed to norfloxacin (71.42%) followed by gentamycin (67.85%) and chloramphenicol (28.57%).

Conclusion: A continuous surveillance for *V. cholerae* is required with respect to changing epidemiology and emergence of antibiotic resistance strains. The source and spread of infection should be investigated to decide the proper management strategies. Additionally, quality of water and status of sanitation should be monitored.

Keywords: Cholera; Vibrio cholera; ElTor biotype; phage type; antimicrobial resistance.

1. INTRODUCTION

Cholera continues to remain a serious public health problem among developing countries like India where the disease is endemic and several outbreaks have been reported [1]. It is the most dreadful acute intestinal infection amongst all diarrhoeal diseases, estimated to affect 3-5 million of people and causes 0.1 million deaths every year. During the 19th century, cholera spread across the world from its original reservoir in the Ganges delta in India. The seventh pandemic started in South Asia in 1961, and reached Africa in 1971 and the Americas in 1991. *V. cholerae* O1 El Tor biotype was the causative agent of seventh pandemic [2]. In 1992, *V. cholerae* O139 was isolated in India and then in Bangladesh. It was the first non-O1 serotype responsible for cholera epidemics. El Tor *V. cholerae* has replaced their classic counterpart over the last few decades [3]. Recently, new variant strains have also been detected in several parts of Asia and Africa. Therefore a careful epidemiological monitoring of circulating strain is recommended [2,4-8].

Cholera is a rapidly spreading disease and has unique epidemiological features. Once endemicity is established in an area, cholera tends to settle into a clear seasonal pattern. The geographical distribution of cholera is changing and so is often considered as a re-emerging disease, in part because infections are appearing in novel communities or in communities where the disease had been absent for many years [9]. This may be due to the changes in environment or climate which affect the occurrence and survival of *V. cholerae*. Additionally, the genetic shifts that are going on in these isolates, adapt them appropriately to survive better in the changing environmental conditions. These also contribute to the increase in drug resistance amongst the *V. cholerae* strains [10-12]. Global replacement of *V. cholerae* classical biotype by El Tor biotype, emergence of O139 serogroup and rapid spread of antibiotic resistant strains indicate the continuous evolution in *V. cholerae* [13].

Spatial and temporal variations in the prevalence of serotypes of *V. cholerae* O1 are usual features and tend to follow a rhythmic pattern, with either of the serotypes dominating at any given time in a given area [3]. Several studies have reported variation in the prevalence of biotypes and serotypes of *V. cholerae* from time to time in different places. The occurrence of cholera has been experienced almost every year during monsoon season in Loni village. Therefore the present study was carried out with the aim to determine biotype, serotype and phage type of *V. cholerae* isolated at Pravara Rural Hospital, Loni. Furthermore, we evaluated the antimicrobial susceptibility pattern of *V. cholerae* isolates.

2. MATERIALS AND METHODS

The present study is part of a Ph.D thesis and was approved by the Institutional Ethics Committee (Registration No. PIMS/PhD/RC/2013/23). The retrospective study was conducted at the Department of Microbiology, Rural Medical College, Pravara Institute of Medical Sciences in Loni, Ahmednagar, Maharashtra, India during January 2009 to December 2012. Stool specimens from patients with acute gastroenteritis were received at Microbiology department. Samples were examined and enriched in alkaline peptone water. Hanging drop preparation was performed to confirm the typical darting motility of *V. cholerae.*

Culturing was then done using BSA (Bile Salt Agar), MacConkey Agar, Blood agar and TCBS (Thiosulphate Citrate Bile Salt Sucrose) agar (Hi-media, Mumbai). The samples were processed according to standard recommended procedures [14]. Biotyping was done according to WHO guidelines and Manual for Laboratory Investigations [15]. Serotyping of isolates was done by seroagglutination using Vibrio polyvalent O1, monospecific Ogawa, Inaba antisera (Central Research Institute, Kasauli). A total of 17 isolates of *V. cholerae* obtained during June 2010 to Dec.2012 were sent to the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, for phage typing and serological confirmation. Phage typing was done by the Vibrio phage reference laboratory of NICED as per earlier published procedures [16,17].

Antibiotic sensitivity testing was done on Muller Hinton agar by disc diffusion method of Kirby and Bauer [18] using ten antimicrobial agents (µg/disc): ampicillin (AMP;10), tetracycline (TE;30), ciprofloxacin (CIP;30) and furazolidone (FZ;50), chloramphenicol (C;10), gentamicin (Gen;10) and co-trimoxazole (CO; 25), norfloxacin (NX; 10), amikacin (AK;10), nalidixic acid (NA;30) (Hi-media, Mumbai). Standard strain of *Escherichia coli* ATCC 25922 was used as control [19].

3. RESULTS AND DISCUSSION

A total of 28 *V. cholerae* were isolated from 544 stool samples of patients with acute gastroenteritis that attended Pravara Rural Hospital during 2009-2012. Out of 28 cholera cases, 13 (46.42%) were male and 15 (53.57%) were female. In endemic regions, the detection rate was reported to vary from 7 to 17%, whereas in outbreak situations it has been shown to increase from 56.5 to 70.3% [20]. The overall detection rate was found to be 5.14% in the present study. The detection rate was 13.33% (10/75) in 2009 followed by 14.67% (16/109) in 2010 which drastically decreased in 2011 as no isolate of *V. cholerae* was detected (0/232) and again in 2012 it was 1.56% (2/128). No major outbreak was observed during the study period. Previously Gujarathi et al. [21] have reported 31.4% detection rate of *V. cholerae* from the same area. Many studies have reported lower rates since 2005 [9,20,22].

The maximum number of cases i.e.10 (35.71%) were found in age group 0-10 yrs followed by 6 (21.42%) in the age group 21-30 yrs and 4 (14.28%) in age group 11-20 yrs. Only 2 cases (7.14%) were recorded in 31-40 age group, 1 (3.57%) in 41-50, 2 (7.14%) in 51-60 and 3 (10.71%) in 61-70 age group. It was seen that both the sexes and all age groups were affected by cholera. These findings are similar with the findings of Jagdish et al. [9]. Several studies have shown a higher prevalence of cholera in children under 5 years of age [12,20,23]. The attack rate or severity of infection depends on many factors such as local

intestinal immunity (which is usually lower in children under age 5 yrs), infectious dose, the adequacy of the gastric-acid barrier, the patient's blood group, quality of water and food and unhygienic conditions [12].

Environmental and climatic factors that drive the seasonality and occurrence of *V. cholerae* and the cholera disease are being increasingly recognized. Cholera cases tend to demonstrate distinct seasonal trends and its occurrence is well correlated with monsoon season, warmest water temperature, heavy rain fall and increased plankton population [10]. The present study showed that every year cholera had seasonality with peak during monsoon i.e. June-September (Fig. 1). These results are in agreement with other studies conducted in different parts of country [9,20,24]. The onset of epidemics coincides with dry climate and the warmest water temperatures of the year i.e. August or September [10,11,25]. In the present study, cholera cases were mainly seen in the month of September (2009) and August (2010 and 2012). The region experienced heavy rain fall during 2009-2010 as compared to 2011-2012. We also report single cases in the month of January and February which are unusual. Similarly, Singh and Khanna reported unusual occurrence of cholera in the month of January and February [26]. This seasonal variation may be due to the onset of monsoon and temperature shifts in this area. Rain increases the level of surface water leading to overflow of wastewater treatment plants or sewer which could contaminate nearby surface waters or wells [10]. Contamination of drinking water with sewage from leaking water pipes and bore-wells leading to possibilities of an epidemic outbreak has been studied [27]. In rural area where hygienic conditions are often compromised, people used such water sources for bathing, cooking and drinking which increases the chances of exposure.

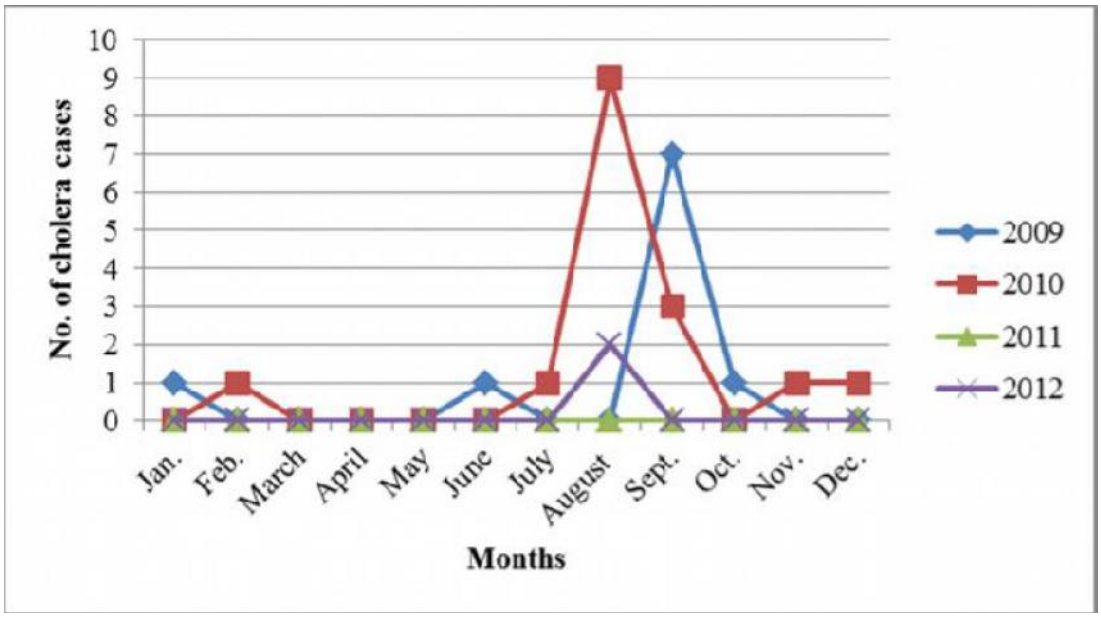

Fig. 1. Seasonal variation of cholera disease during 2009-2012

All 28 isolates were identified as *V. cholerae* O1 El Tor biotype on the basis of Voges-Proskauer (VP) test, polymyxin B sensitivity, haemolysis and haemagglutination test [15]. All strains were non haemolytic, resistant to polymyxin B, VP positive and showed

haemagglutination with sheep erythrocyte. Saini et al. [28] in 1991 also observed similar results for biotyping of Vibrio strains isolated in Haryana [. All these isolates belonged to sub-serotype Ogawa strain. Inaba and Hikojima sub serotypes were not found in this study. No isolate of *V. cholerae* O139 was detected. These results are similar to that of Priya D *et al* [22]. In 1994 Jain et al. [29,21] reported occurrence of Inaba sub serotype in this area, but since 1996 no isolate belonging to Inaba serotype was reported. This change probably could be the result of genetic assortment and re-assortments take place in the isolates to adapt them in the changing environment [9-11].

17 isolates obtained during June 2010 - Dec. 2012 were sent to NICED, Kolkata for phage typing and serotyping confirmation (Table 1). The NICED result showed that all isolates were *Vibrio cholerae* O1, biotype El Tor, serotype Ogawa. Phage typing was done using Basu-Mukerjee and New phage typing schemes at NICED. All these isolates belonged to phage type T-2 as per Basu – Mukerjee scheme [16]. According to the new scheme for phage typing developed by NICED [17], 12 isolates were of phage type T-27, 2 were T-13 and 1 was T-26. Out of total 15 isolates obtained in the year 2010 only 13 isolates were typed by both phage typing schemes and 2 were untypeable. Prior to this, phage type T4 was prevalent in this area [29] and multiple phage types have been previously reported [21]. A new phage type T-13 was observed in this study. Most of the studies have done phage typing using Basu – Mukerjee scheme and new phage typing scheme developed at NICED [9,20,28]. Sharma et al. [20] also found T-27 type as the prevalent phage type in Delhi, however phage types 4, 16, 25 (on the basis of new phage typing scheme) were prevalent in other parts of the country. Jagdish et al. [9] reported multiple phage types including T-27 as the prevalent type followed by T-26. Overall country wise epidemiological data reports type 27 to be the predominant phage type. The distribution of a common type throughout the country suggests that a particular clone of *V. cholerae* O1 is probably circulating all over India [30]. Recently, Sarkar et al. [31] have done phage typing of *V. cholerae* O1 and O139 (1998–2007) strains received from different parts of India for the identification of trends in the occurrence and spread of cholera in the country. The study revealed that *V. cholerae* O1 (T-27) and O139 (T-1) strains circulate throughout the country at any given time.

Increasing antimicrobial resistance in diarrhoeagenic microorganisms is becoming a major problem worldwide. Drug resistance complicates the treatment and puts the extra unaffordable burden of newer expensive drugs on the population of developing countries [32,33]. There is great variation in the distribution of antibiotic resistant *V. cholerae* O1 strains worldwide [34]. In the present study, 100% resistance for co-trimoxazole, nalidixic acid and ampicillin was observed among *V. cholerae* isolates and 50% of the isolates were resistant to furazolidone (Table 2). A study from Calcutta reported an increase in resistance to ampicillin, co-trimoxazole, and nalidixic acid from the year 1994 onwards [35]. Inacio et al. [36] have reported similar resistance to co-trimoxazole. Similar significant increase in resistant strains was observed against nalidixic acid by Urassa et al. [37]. Antibiogram of *V. cholerae* O1 isolates collected from different cholera outbreaks in different regions of India between 2004 and 2007 was studied by Goel et al. [4] and revealed resistance towards several antibiotics including nalidixic acid, co-trimoxazole, streptomycin, nitrofurantoin and polymyxin B. However, antibiogram of the strains confirmed susceptibility to tetracycline and chloramphenicol in all the isolates. Though tetracycline is the drug of choice for treating cholera, we have observed 28.57% resistance to the drug during study period. Emergence of tetracycline resistant strains have also been reported by Mhalu et al. [38] and Bhattacharya et al. [32].

Table 1. Phage typing of *Vibrio cholerae* isolates year 2010-2012

Year	*Vibrio cholerae* Type	Total number of cholera cases recorded	Total number of Vibrio isolates subjected for phage typing	Total number of isolates typed	Total number of isolates untypeable (UT)	Phage Type (Number of isolates) based on Basu and Mukherjee Scheme	Phage Type (Number of isolates) based on new phage typing scheme
2010	*Vibrio cholerae* Biotype- ElTor Serotype- Ogawa	16	15	13	2	T-2 (13) T-4 (0)	T-27 (12) T-13 (1)
2011	-	-	-	-	-	-	-
2012	*Vibrio cholerae* Biotype- ElTor Serotype- Ogawa	2	2	2	0	T-2 (2) T-4 (0)	T-26 (1) T-13 (1)
Total		**18**	**17**	**15**	**2**	**15**	**15**

Isolates were not phage- typed during the period Jan.2009-Jan.2010 (Total 10 isolates of V. cholerae El Tor Ogawa)

Remarkable increase in tetracycline-resistant *V. cholerae* O1 strains was observed during major epidemics of Latin America, Tanzania, Bangladesh, and Zaire [35]. In the present work, maximum sensitivity was observed to norfloxacin (71.42%) followed by gentamycin (67.85%), chloramphenicol (28.57%) and ciprofloxacin (21.42%). Similarly, Saini et al. [39] also found maximum sensitivity to norfloxacin and gentamycin. However, increased resistance to newer fluoroquinolones, such as ciprofloxacin and norfloxacin, among *V. cholerae* strains belonging to O1 serogroup has also been reported [40]. Recently, Kutar et al. [41] analysed antibiotic resistance phenotype of *V. cholerae* isolates from Kolkata and showed that all isolates were sensitive to gentamycin but few isolates were resistant to norfloxacin (1.7%) and kanamycin (3.4%). The variation in drug resistance pattern of isolates can be the result of different antibiotic resistance mechanisms. Several studies have been conducted to unravel the antibiotic resistance mechanism of *V. cholerae* and genetic factors including efflux pumps, spontaneous chromosomal mutation, conjugative plasmids, SXT elements and integrons, have been evaluated to understand their role in conferring antibiotic resistance [42]. The SXT related ICE's (Integrative conjugative element) which accounted for antibiotic resistance phenotype has been characterized and their prevalence in various clinical isolates of *V. cholerae* from India was reported [41,43]. It has been shown that the presence of SXT can be accounted for co-trimoxazole resistance whereas resistance to nalidixic acid can be the result of mutation in topoisomerases [41]. The characteristic similarity between isolates from India, Bangladesh and Haiti outbreak indicate circulation of SXT containing *V. cholerae* in all these areas which contribute in the observed drug resistance profile of isolates [41]. In the present study, the number of isolates is too few to make any valid comparison for analysing changing trends in antibiotic resistance pattern.

Table 2. Antimicrobial sensitivity pattern of *Vibrio cholerae* isolates

Sr. No.	Antibiotics	No.(%) of *Vibrio cholerae* sensitive/resistant to selected antibiotics during 2009-2012 Total number of isolates=28		
		Resistant (%)	Intermediate (%)	Sensitive (%)
1.	Chloramphenicol (C10)	14 (50)	6 (21.42)	8 (28.57)
2.	Ciprofloxacin (CIP30)	9 (32.14)	13 (46.42)	6 (21.42)
3.	Tetracycline (TE30)	8 (28.57)	13 (46.42)	7 (25)
4.	Nalidixic Acid (NA30)	28 (100)	0 (0)	0 (0)
5.	Ampicillin (AMP10)	28 (100)	0 (0)	0 (0)
6.	Gentamycin (Gen10)	5 (17.85)	4 (14.28)	19 (67.85)
7.	Norfloxacin (NX10)	5 (17.85)	3 (10.71)	20 (71.42)
8.	Co-trimoxazole (CO25)	28 (100)	0 (0)	0 (0)
9.	Amikacin (AK10)	13 (46.42)	5 (17.85)	10 (35.71)
10.	Furazolidone (FZ50)	14 (50)	8 (28.57)	6 (21.42)

4. CONCLUSION

In conclusion, the present study documented bacteriological profile and antibiogram of *V. cholerae* in the rural set up of Loni village of Ahmednagar district, Western Maharashtra. A continuous long term surveillance programme is required to prevent the occurrence of cholera and to monitor changing patterns of antimicrobial resistance of isolates. Further genotypic characterization of *V. cholerae* O1 isolates needs to be done. Additionally, other factors such as source of infection, quality of water and status of sanitation should be monitored.

ETHICAL APPROVAL

The present work was approved by the Institutional Ethics Committee (Registration No. PIMS/PhD/RC/2013/23).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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