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Prevalence of multidrug resistant altered *Vibrio cholerae* O1 isolates among Diarrhoeal patients in Delhi during 2008-2012.

KEYWORDS: *V. cholerae* O1, VP test, D-MAMA PCR, Antibiotic susceptibility test, Phage type.

Puneeta Singh	Dhirendra Kumar	Yogendra Prasad
Department of Aquatic Biotechnology, & Fisheries, MJP Rohilkhand University, Bareilly	Laboratory Department, Maharishi Valmiki Infectious, Diseases Hospital, Delhi, India	Department of Aquatic Biotechnology, & Fisheries, MJP Rohilkhand University, Bareilly

Thandavarayan Ramamurthy	Banwari Lal Sarkar	Naresh Chand Sharma
National Institute of cholera & Enteric Diseases, Kolkata	National Institute Of Cholera & Enteric Diseases, Kolkata	Maharishi Valmiki Infectious Diseases Hospital, Delhi

ABSTRACT *Altered Vibrio cholerae O1 has been reported from some areas in the country as well as from the other countries. Delhi is an endemic area for toxigenic V. cholerae O1. Therefore, we were interested to evaluate the presence of altered Vibrios, their drug resistance status and phage types among the strains isolated from suspected cholera/acute gastroenteritis patients admitted in this hospital. A total of 59 isolates representing different years were used for D -MAMA PCR to detect the Haitian genotype (ctxB7) strains and classical genotype (ctxB1). A subset of 49 isolates was tested to determine the phage types. All selected strains were serologically positive for V. cholerae O1, serotype Ogawa and biochemically biotype El Tor. The antibiotic susceptibility test explicated that 100% strains were resistant to cotrimoxazole and nalidixic acid. Phage typing revealed that 79.6% of isolates were phage type T-27. D-MAMA PCR, has shown 52.5% strains were carrying ctxB7 gene since 2008.*

Introduction

Vibrio cholerae O1 biotype El Tor is an enteric bacterial pathogen causing cholera disease in many parts of Asia and Africa (WHO, 2011). It is an immense public health concern owing to its potential to produce pandemics of cholera disease in human population (Emch *et al*., 2008). The cholera disease, caused by O1 or O139 serogroups of *V. cholerae* is generally marked with rapid loss of body fluids leading to dehydration, electrolyte disturbances and hypovolemic shock, In the absence of treatment death may occur within hours (Sack *et al.,* 2004). Recent data have shown an expansion of *V. cholerae* in different parts of India, as its various strains poses great threat being endemic (Samal *et al*., 2008, Das *et al*., 2008, Ramamurthy & Sharma, 2014). Developing countries like India still endure frequent outbreaks of cholera through water or food which are the main source of this pathogen in the absence of basic sanitation services and clean water (Nair *et al*., 2007). Since the first occurrence of *V. cholerae* O1 biotype El Tor during 1964 in India and Delhi in June 1965, the constant endemicity of cholera has been noted throughout the year in Delhi (Sharma *et al*., 2007) indicating the transformation of disease from rare prevalence to seasonal to annual (Nair, 2007). *V. cholerae* O1 biotype El Tor serotype Ogawa has always been the predominant serovar causing outbreaks in different areas of Delhi and other states except during 2004 to 2006. However, prevalence of both Ogawa and Inaba serotype in Delhi and other parts of India has also been noted (Dutta *et al*., 2006; Das *et al*., 2008 and Rajeshwari *et al*., 2008).

The emergence of multiple drugs resistant isolates of *V. cholerae* (Sharma *et al*., 2007; Kitaoka *et al*., 2011; Kuma *et al*., 2014) and changing pattern of antibiogram has increased interest among the scientists to reconsider the role of antibiotics in cholera epidemics. Moreover, resistance to commonly used antibiotics viz. ampicillin, tetracycline, ciprofloxacin, chloramphenicol, ceftriaxone has been found in *V.cholerae*. The El Tor biotype is known to be more infective but less virulent than the classical biotype, therefore the El Tor strains that produce the classical cholera toxin is a new emerging form that appears to be causal of a more severe disease (Ghosh- Banerjee *et al.,* 2010).

In Delhi, our study showed strains of *V. cholerae* O1 belong to the altered El Tor, indicating that a cryptic change has occurred in the seventh pandemic El Tor biotype strains of *V. cholerae* O1. Therefore, this study was planned to evaluate antibiotic sensitivity of these strains and to develop a PCR based diagnostic test that could distinguish between two kinds of *ctxB* genes to provide a means of monitoring the presence and dissemination of the new emerging variants of *V. cholerae* O1 of the El Tor biotype in Delhi.

2. Materials and Methods:

2.1 Bacterial Strains and biochemical & Serological Characterization:

Fresh stool specimens were obtained from patients admitted in the Maharishi Valmiki Infectious Diseases Hospital, Kingsway Camp, Delhi in 2008-2012. None had received antibiotic treatment prior to collection of the stool specimens. *V. cholerae* O1 positive 150 representative samples from the outbreaks were collected for investigation and analysis. All samples were stored in Cary Blair's transport medium, transported at ambient temperature to the laboratory within 1 hour and then plated directly on thiosulfate citrate bile salt sucrose (TCBS) agar and alkaline bile salt (BSA) agar (Himedia Mumbai, India). All strains with sucrose fermenting yellow colonies on TCBS agar and oxidase positive were further subjected to conventional biochemical tests for confirmation of *V. cholerae* as per standard protocol (CDC/WHO/USAID, 2003). All strains were biotyped by using standard procedures including susceptibility test to 50 units of polymixin B, chicken cell erythrocytes and VP test, using strains 569B & NI6961 as

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the classical & El Tor reference strains respectively (Kaper *et al.,* 1995). All strains were subjected to serogrouping and serotyping by serogroup specific O1 polyvalent antiserum and serotype specific antiserum for Ogawa & Inaba strains, respectively (Denka Seiken, Tokyo, Japan).

2.2 Antimicrobial Susceptibility Testing:
All isolates of *V. cholerae* O1 El Tor Ogawa were tested for antimicrobial susceptibility using the disk diffusion method according to Clinical & Laboratory Standard Institute (CLSI) guidelines with commercial antibiotic discs (Hi-Media-Mumbai, India) containing ampicillin (10µg), chloramphenicol (30 µg), ciprofloxacin (5µg) gentamicin (10µg), levofloxacin (5µg), nalidixic acid(30µg), norfloxacin (10µg), streptomycin (10µg), tetracycline (30µg), trimethoprim (5µg), ceftriaxone (30µg), azithromycin (15µg), sparfloxacin (5µg), ofloxacin (5µg), furazolidone (100µg), doxycycline (30µg), cotrimoxazole (25µg). Susceptibility tests for polymixin B 50 Units in nutrient agar (Hi-media, Mumbai, India) were carried out separately by standard methods (CDC/ WHO/USAID, Manual, 2003). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for disc diffusion.

Isolation of genomic DNA
Overnight cultures of *V. cholerae* O1 isolates grown on Luria Agar (LA) were used for genomic DNA extraction. One loopful growth harvested from LA plate was suspended into TE buffer. After vortexing the suspension was boiled in a water bath at 100°C for 10 min and chilled in ice for 5 min followed by centrifugation at 10,000 rpm for 5 min. Supernatants were used as the genomic DNA templates (Borkakoty *et al.,* 2012).

Determination of *ctxB*1 and Haitian *ctxB*7 genes of *V. cholerae* O1 strains by double mismatch amplification mutation assay (D-MAMA) PCR.
The strains were examined by double mismatch amplification mutation assay (D-MAMA) PCR for detecting the *ctxB* allele using a common reverse primer for two alleles ReCla(5'CCTGGTACTTCTACTTGAAACG 3') and two allele specific primers *ctxB*-F3(5'GTTTTACTATCTTCAGCATATGC GA3'), and *ctxB*-F4(5'GTTTTACTATCTTCAGCATATGCGC 3') for Haitian and classical, respectively (Naha *et al.*, 2012).

Phage Typing
A subset of 49 strains of *Vibrio cholerae* O1 was sent to the National Institute of Cholera and Enteric Diseases (NICED), Kolkata for the phage typing. Phage typing was done as per procedures published earlier (Basu and Mukerjee., 1968; Chattopdhyay DJ *et al*., 1993)

3. Results:
All 150 isolates confirmed biochemical features and serologically *V. cholerae* serogroup O1 biotype El Tor serotype Ogawa. Result of AST revealed that 100% strains were sensitive to azithromycin, gentamicin, levofloxacin, ofloxacin and norfloxacin while 99.04%, 96%, 92% & 88% strains were susceptible to ceftriaxone, chloramphenicol, doxycycline and tetracycline, respectively. Conversely, all 150 strains (100%) were resistant to co-triamoxazole and nalidixic acid while 97%, 93%, 90%, 32.2% and 17.3% strains were resistant to trimethoprim, streptomycin, furazolidone, ampicilin and ciprofloxacin respectively (Table 1). The result of phage typing revealed that 79.6% of isolates were T 27 while 4% each were of T 19 and T 23 only 2 % were of T 5 types rest were untypable (Table -2). Interestingly, the D-MAMA PCR performed for 59 strains showed the presence of Haitian (*ctxB*7) (52.5%) (Fig 1a), as well as classical (*ctxB*1) (11.86%) (Fig 1b) variants.

Discussion :
All pandemics were caused by classical biotype till sixth pandemic but seventh pandemic (1961-till date) was caused by El Tor biotype and continuously caused epidemics in many countries (Ramamurthy & Sharma, 2014). In recent years, the emergence of many pathogenic variants of *V. cholerae* O1 in many Asian and African countries was recorded (Alam *et al*., 2010, Chin *et al*., 2011, Tran *et al*., 2012, Kumar *et al*., 2013, Kuma *et al.,* 2014) indicated an epidemiology of cholera being changed. Multiple antibiotic resistant *V. cholerae* O1 has been detected from patients in MVID Hospital, Delhi from different areas during 2008-2012. Tetracycline is first line of effective drug for treatment of cholera (WHO, 2005). Previously many investigators had reported tetracycline resistance among multidrug resistant *V. cholerae* O1 (Samal *et al,* 2008, Sharma *et al*., 2007, Das *et al.,* 2008, Sharma *et al*., 2015). An earlier study from this hospital recorded the circulating *V. cholerae* O1 El Tor Ogawa and Inaba strains resistant to multiple drugs viz. tetracycline, nalidixic acid, furazolidone, chloramphenicol (Dutta *et al*., 2006, Sharma *et al*., 2007, Taneja *et al.,* 2005). Ciprofloxacin resistant strains have been circulating and this was reported by many workers and our findings are in agreement with other reports (Pal *et al.,* 2010, Borkakoty *et al.,* 2012, Bhattacharya *et al.,* 2011, Kumar *et al*., 2013). All Haitian variants are resistant to ampicillin, nalidixic acid, cotrimoxazole, streptomycin, trimethoprim, furazolidone and some strains are ciprofloxacin resistant in present study. Many challenges are arising due to increased resistance of these drugs since many cost effective drugs are out of reach for poor people and in developing countries this has complicated the control strategies of cholera disease and moreover, result in longer hospital stay for patients. Trimethoprim, furazolidone, streptomycin, nalidixic acid and cotrimoxazole are 85-100% resistant during these years. In our study only one strain was found resistant to ceftriaxone and six strains to chloramphenicol. In this study azithromycin and levofloxacin were found sensitive hence proposed for the treatment of pregnant women and pediatric age group suffering from *V. cholerae* O1 infection. Phage typing results of 49 *V. cholerae* O1 strains on the basis of new phage typing scheme (Chattopdhyay *et al*., 1993) showed phage type T27 (79.6%) to be the most common type in Delhi in the present study and similar results were reported by other workers in Delhi (Das *et al.,* 2005, Sharma *et al*., 2007).

Phylogenies for whole genome sequences showed that the Haiti outbreak strain is genetically related to strains originating in Nepal, India and Cameroon (Reimer *et al*., 2011, Son *et al.,* 2011). Haiti isolate harbours variant of the classical *ctxB* gene (*ctxB7*). The *ctxB* gene variant *ctxB*7 was first observed from Orissa, India, in 2007 (Goel *et al*., 2008). The strains which are carrying *ctxB*7 outbreak are already present in Delhi, India since 2008, and this genotype was already reported from other parts of the country (Goel *et al*., 2008, Naha *et al.,* 2012, Kutar *et al*., 2013, Kumar *et al*., 2014). Previous investigation showed that Haitian variant with higher fitness virulence and antimicrobial drug resistant phenotypes, the Haitian variants harbours infectivity and ecologic persistence advantage over the other seventh pandemic strains (Riemer *et al*., 2011). It appears that the Haitian variant has replaced the altered El Tor *Vibrio cholerae* O1 strains circulating in Delhi. Such direct prediction enhanced epidemiologic surveillance and will help in deciding priorities aimed at cholera prevention. In present

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set of 59 strains 31 (52.5%) were found carrying *ctxB7*. In an another report from Kolkata, India 93% isolates were reported to had *ctxB7* gene in 2011 (Naha *et al.*, 2012) whereas Kutar *et al.*, (2013) found only 46.2% strains having *ctxB7* gene. However in a recent report from Yavatamal and Kalamb, Maharashtra 100% strains were found to contain *ctxB7* gene (Kumar *et al.*, 2014). These reports clearly indicate that *V. cholerae* O1 strains carry *ctxB7* are replacing *V. cholerae* O1 carrying El Tor or altered *ctxB* gene.

Conclusion: The results obtained in the present study have demonstrated the emergence of multidrug resistant strains, emergence of newly encountered. Phage types and changes in the *ctxB* gene, there is urgent need to monitor the said characteristics and adopt revised strategies to control cholera disease in Delhi.

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Table1. Antibiotic resistance pattern of *Vibrio cholerae* isolates (n = 150)

Antibiotics	Serotypes Ogawa (n-150)	Resistance Overall (n-150)	Antibiotics	Serotypes Ogawa (n-150)	Resistance Overall (n-150)
Ampicilin	49/150	32.2%	Nalidixic acid	150/150	100%
Azithromycin	0/150	0%	Streptomycin	140/150	93%
Chloramphenicol	6/150	4%	Tetracycline	18/150	12%
Co-triamoxazole	150/150	100%	Trimethoprim	146/150	97%
Ceftriazone	1/150	0.06%	Levofloxacin	0/150	0%
Ciprofloxacin	26/150	17.3%	Ofloxacin	0/150	0%
Doxyclycline	12/150	8%	Norfloxacin	0/150	0%
Gentamicin	0/150	0%			
Furazolidone	136/150	90%			

Table –2: Phenotypic characteristics of *V. cholerae* O1 strains (n=49) isolated from Clinical samples collected in Delhi (2008-2011)

Sl no	Place	Year	VC O1 Strain no	SEROLOGY	VP Test	Poly B (50U)	Chick cell aglu	Phage type Basu & Mukherje	Phage type new scheme
1	Delhi	2008	8169	Ogawa	+	R	+	T-2	T-27
2	Delhi	2008	8227	Ogawa	+	R	+	T-2	T-27
3	Delhi	2008	8369	Ogawa	+	R	+	T-2	T-27
4	Delhi	2008	8443	Ogawa	+	R	+	T-2	T-27
5	Delhi	2008	8930	Ogawa	+	R	+	T-2	T-27
6	Delhi	2008	9582	Ogawa	+	R	+	T-2	T-27
7	Delhi	2008	9995	Ogawa	+	R	+	UT	UT
8	Delhi	2008	10285	Ogawa	+	R	+	T-2	T-27
9	Delhi	2008	10328	Ogawa	+	R	+	T-2	T-27
10	Delhi	2008	10345	Ogawa	+	R	+	T-2	T-27
11	Delhi	2009	12535	Ogawa	+	R	+	T-2	T-27
12	Delhi	2009	13359	Ogawa	+	R	+	T-2	T-5
13	Delhi	2009	13596	Ogawa	+	R	+	T-2	T-27
14	Delhi	2009	13856	Ogawa	+	R	+	T-2	T-27
15	Delhi	2009	13799	Ogawa	+	R	+	T-2	T-27
16	Delhi	2009	14010	Ogawa	+	R	+	T-2	T-27
17	Delhi	2009	14463	Ogawa	+	R	+	T-2	T-27
18	Delhi	2009	14464	Ogawa	+	R	+	T-2	T-27
19	Delhi	2009	14581	Ogawa	+	R	+	UT	UT
20	Delhi	2009	15468	Ogawa	+	R	+	T-2	T-27
21	Delhi	2009	16368	Ogawa	+	R	+	T-2	T-27
22	Delhi	2010	20370	Ogawa	+	R	+	T-2	T-27
23	Delhi	2010	20371	Ogawa	+	R	+	T-2	T-23

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24	Delhi	2010	20956	Ogawa	+	R	+	T-2	T-27
25	Delhi	2010	23979	Ogawa	+	R	+	T-2	T-27
26	Delhi	2010	23485	Ogawa	+	R	+	T-2	T-27
27	Delhi	2010	23678	Ogawa	+	R	+	UT	UT
28	Delhi	2010	23855	Ogawa	+	R	+	T-2	T-27
29	Delhi	2010	23731	Ogawa	+	R	+	T-2	T-27
30	Delhi	2010	23835	Ogawa	+	R	+	T-2	T-27
31	Delhi	2010	23902	Ogawa	+	R	+	T-2	T-27
32	Delhi	2010	20893	Ogawa	+	R	+	T-2	T-27
33	Delhi	2010	20045	Ogawa	+	R	+	T-2	T-23
34	Delhi	2011	26891	Ogawa	+	R	+	T-2	T-27
35	Delhi	2011	27071	Ogawa	+	R	+	T-2	T-19
36	Delhi	2011	27121	Ogawa	+	R	+	T-2	T-26
37	Delhi	2011	27122	Ogawa	+	R	+	T-2	T-27
38	Delhi	2011	27161	Ogawa	+	R	+	T-2	T-27
39	Delhi	2011	26973	Ogawa	+	R	+	T-2	T-27
40	Delhi	2011	26874	Ogawa	+	R	+	T-2	T-27
41	Delhi	2011	26871	Ogawa	+	R	+	T-2	T-27
42	Delhi	2011	26975	Ogawa	+	R	+	T-2	T-27
43	Delhi	2011	26976	Ogawa	+	R	+	T-2	T-27
44	Delhi	2011	26866	Ogawa	+	R	+	T-2	T-27
45	Delhi	2011	27174	Ogawa	+	R	+	T-2	T-27
46	Delhi	2011	26903	Ogawa	+	R	+	UT	UT
47	Delhi	2011	26904	Ogawa	+	R	+	T-2	T-27
48	Delhi	2011	26907	Ogawa	+	R	+	T-2	T-19
49	Delhi	2011	27169	Ogawa	+	R	+	Do	T-27

The result of phage typing (Table -3) revealed that 79.6% of isolates were T-27 while 4% were of T19 and T 23 only 2 % were of T 5 and rest were UT 8% types as diagnosed in phage typing new scheme. VP test-Voges Pauskauer test, Chick cell aggl- Chicken cell agglutinate, poly B- Polymixin B

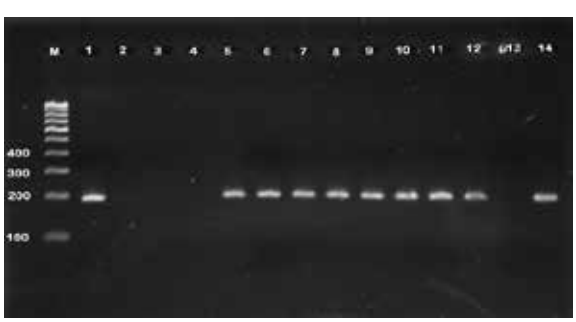

Figure 1a; PCR with F3-*ctxB*Cla (Rev)

Agarose gel (1.5%), Lane M 100bp Molecular weight marker, Lane no 1,(IDH00745)Haitian control strain, 2(0395)classical control, 3(N16961) El-Tor control, 4 (Negative control),5,6,7,8,9,10,11,12,13,14 (test strains).

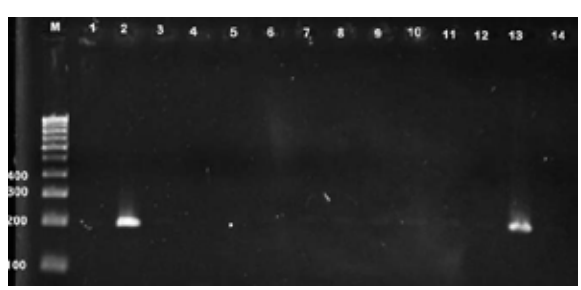

Figure 1b; PCR with F4-*ctxB*Cla (Rev)

Agarose gel (1.5%), Lane M 100bp Molecular weight marker, Lane no 1,(IDH00745)Haitian control strain, 2(0395)classical control, 3(N16961) El-Tor control, 4 (Negative control),5,6,7,8,9,10,11,12,13,14 (test strains).

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