Molecular Characterization of *Vibrio cholerae* O1 Reveals Continuous Evolution of Its New Variants in India

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Abstract *Vibrio cholerae*, the causing agent of cholera is still a major health challenge in most of the developing countries. In this study, *V. cholerae* strains collected from different cholera outbreaks in India over a period of past 7 years were found to have various toxigenic, pathogenic and regulatory genes viz. *ctxAB*, *zot*, *tcp*, *hlyA*, *ace*, *ompU*, *ompW*, *rfbO1*, *toxT* and *toxR*. The biotype specific genes *rstR* and *rtxC* revealed the El Tor biotype in majority of the isolates. However, variants among the isolates were found having genotype of both the biotypes. Sequencing of *ctxB* gene revealed the presence of altered *ctxB* of classical biotype with additional variations in isolates of 2007. Mismatch amplification mutation assay PCR also confirmed the isolates belonging to classical biotype. Antibigram of the isolates revealed resistance for nalidixic acid, co-trimoxazole, streptomycin, and polymyxin B and susceptibility for tetracycline among most of the isolates from India. However, *V. cholerae* isolates from a recent outbreak in Eastern India were resistant to tetracycline. The study corroborated the continuous emergence and wide-spread of multidrug resistant El Tor variant strains in the Indian subcontinent.

Keywords Cholera · Evolution · Outbreak · Toxigenic · *Vibrio cholerae*

Introduction

Cholera is a fatal waterborne gastroenteritis disease extensively recorded in human history since ancient times. Mostly it is associated with sub-standard sanitation and unsafe drinking

water. WHO recorded a 85 % increase in cholera cases in 2011 as compared to 2010 with 589,854 cases including 7,816 deaths with a case fatality rate of 1.3 % from 58 countries [1]. These figures actually represent the tip of iceberg because of underreporting and other limitations of surveillance systems. The disease is caused by *Vibrio cholerae*, a Gram-negative bacterium, encompassing more than 200 serogroups based on ‘O’ antigen of lipopolysaccharide. However, strains belonging to O1 and O139 serogroup only harness the epidemic and pandemic potential. There are two biotypes of *V. cholerae* O1, classical and El Tor. The first six pandemics were caused by classical biotype strains, which are suggested to be more toxigenic than El Tor strains. However, seventh pandemic was caused by El Tor biotype strains associated more frequently with asymptomatic infections and fewer fatalities but with better adaptability to survive in the environment and in the human host than classical biotype. Recently, new variants of *V. cholerae* O1 having traits of both the classical and El Tor biotypes have been seen [2–4].

Vibrio cholerae strains with multiple antibiotic resistance have been observed among the outbreak strains [5, 6]. The emergence of drug resistant *V. cholerae* is a big challenge for health authorities in disease management. The present study was aimed at genetic analysis and antibiogram study of *V. cholerae* strains collected from different cholera outbreaks in India since 2004 for monitoring of any new changes among the isolates.

Materials and Methods

Bacterial Cultures

A total of 216 *V. cholerae* O1 strains collected from clinical samples during the past 7 years (2004–2010) from

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cholera outbreaks at various locations in India were taken into study (Table 1).

Biochemical Characterization

The bacterial isolates were screened for oxidase reaction followed by standard biochemical tests for presumptive identification of *V. cholerae* [7]. Isolates were serotyped with polyvalent antiserum against *V. cholerae* O1 (Ogawa and Inaba) and O139 (Difco Laboratories, Detroit, MI). The isolates were subjected to Voges Proskauer (VP) test, sheep erythrocyte haemolysis and polymyxin B susceptibility tests for determination of biotype.

PCR Analysis of Isolates

Bacterial genomic DNA was extracted from the isolates using genomic DNA purification kit (MBI Fermentas, Vilnius, Lithuania). The confirmation of *V. cholerae* and its serogroup, toxigenicity and pathogenicity traits were assayed by two sets of multiplex PCR (mPCR) as described previously [8]. The first mPCR confirmed the presence of genes encoding the outer-membrane protein (*ompW*), cholera toxin (*ctxAB*), zonula occludens protein (*zot*), O1 somatic antigen (*rfbO1*) and toxin coregulated pilus (*tcp*). The second mPCR detected the other virulence and toxigenic genes encoding the accessory cholera enterotoxin (*ace*), haemolysin (*hlyA*), outer membrane protein (*ompU*), repeat in toxin protein (*rtxC*) and toxin regulator (*toxR*). PCR primers used for amplification of different genes along with their amplicon sizes used in this study are described elsewhere [8].

PCR for Biotyping of Isolates

PCR was performed using the allele specific *rstR* primers, *rstR*^{El Tor} and *rstR*^{Classical} for detection of allelic types of *rstR* in clinical isolates of *V. cholerae* as described previously [9]. For detection of biotype specific *ctxB* allele, the isolates were subjected to mismatch amplification mutation assay (MAMA) PCR as described elsewhere [10]. Additionally, randomly selected isolates were subjected for *ctxB* gene sequencing as described earlier [5].

Antibiogram

Vibrio cholerae isolates were tested for antimicrobial drug susceptibility by disk diffusion assay on Mueller–Hinton agar as per standard procedure. The antibiotics impregnated disks (Oxoid Ltd, Hants, UK) used were: ampicillin, 10 µg; cefixime, 5 µg; chloramphenicol, 30 µg; clindamycin, 2 µg; co-trimoxazole, 25 µg; doxycycline, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; nalidixic acid, 30 µg; neomycin,

30 µg; norfloxacin, 10 µg; ofloxacin, 5 µg; polymyxin B, 300 U; rifampicin, 5 µg; spectinomycin, 100 µg; streptomycin, 10 µg; sulfamethoxazole, 100 µg; tetracycline, 30 µg; trimethoprim, 5 µg; and the vibriostatic agent O/129, 150 µg.

Results and Discussion

Bacterial Identification

A total of 216 *V. cholerae* isolated from the affected patients of cholera outbreaks from different places of India between 2004 and 2010 were biochemically identified as *V. cholerae* and serologically confirmed as O1 Ogawa.

Distribution of Virulent Genes Among the Isolates

Two sets of mPCR revealed that all the outbreak isolates were positive for various virulent and other genes viz. *ace*, *ctxAB*, *hlyA*, *ompU*, *ompW*, *rfbO1*, *rtxC*, *tcpA*, *toxR* and *zot* genes (Table 1). The *ompW* gene contributes significantly in the outer membrane protein profile of bacteria and it is species specific for *V. cholerae* [11, 12]. Somatic O-antigen biosynthesis gene was positive for O1 type (*rfbO1*), providing molecular evidence for O1 serogroup. The presence of the *ctxAB* gene confirmed the toxigenicity of the isolates. Among the various toxins produced by *V. cholerae*, cholera toxin (CT), an A-B type toxin is the principle virulence factor and serves as a marker of epidemic potential [13]. The *ctxAB* operon resides in the genome of CTXΦ, which is further divided into the core and RS2 regions [14]. The core region of 4.5 kb possesses *ctxAB*, *zot*, *ace*, *orfU*, and *cep* genes and the RS2 region of 2.6 kb encodes proteins for replication, integration and regulation of CTXΦ [14]. The acquisition of CTXΦ relies on the presence of toxin co-regulated pilus (encoded by *tcp* genes) on cell surface, which acts as a receptor for CTXΦ [14]. The zonula occludens and accessory CTs are also virulence factors in *V. cholerae*. These toxigenic and pathogenic genes have been invariably found in all the outbreak *V. cholerae* isolates from India [8, 15]. Water, especially contaminated drinking water harbours various toxigenic *V. cholerae* and plays an important role in transmission of cholera [16, 17].

Biotyping of Isolates

Determination of the classical and El Tor biotypes within the O1 serogroup relies on conventional biochemical and genetic analyses. On the basis of biochemical tests, all the isolates were found to belong to El Tor biotype as the isolates were VP test positive, hemolytic on sheep blood agar and resistant to polymyxin B.

Table 1 Biotype and genotypic characteristics of outbreak *V. cholerae* isolates

Year of isolation	Location of isolation	Number of strains	Strain type	Biotype characteristics				Genotype			Antibiogram		
				test				MAMA PCR	ctxB sequence	Multiplex PCR1		Multiplex PCR2	
				VP	PB	Haemolysis	rtxC						rstR
1948	Classical 569B, India	1	O1 Classical	-	S	-	-	C	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁻ ace ⁺ hlyA ⁻ ompU ⁺ toxR ⁺	
1971	El Tor N16961, Bangladesh	1	O1 El Tor	+	R	+	+	E	E	20 (H), 39 (Y), 68 (I)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	
2004	Chennai, Tamil Nadu	44	O1 El Tor	+	R	+	+	E	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2005	Chennai, Tamil Nadu	38	O1 El Tor	+	R	+	+	E	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2007	Rayagada, Orissa	18	O1 El Tor	+	R	+	+	E	C	20 (N), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2007	Kalahandi, Orissa	6	O1 El Tor	+	R	+	+	E	C	20 (N), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2007	Koraput, Orissa	8	O1 El Tor	+	R	+	+	E	C	20 (N), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2009	Hyderabad, Andhra Pradesh	33	O1 El Tor	+	R	+	+	E	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2009	Hyderabad, Andhra Pradesh	1	O1 El Tor	+	R	+	+	E + C	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2010	Solapur, Maharashtra	41	O1 El Tor	+	R	+	+	E	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tri, O129
2010	Rayagada, Orissa	27	O1 El Tor	+	R	+	+	E	C	20 (H), 39 (H), 68 (T)	ompW ⁺ ctxAB ⁺ rfbO1 ⁺ tcp ⁺ zot ⁺	rtxC ⁺ ace ⁺ hlyA ⁺ ompU ⁺ toxR ⁺	Na, PB, Str, Sul, Tet, Tri, O129

H histidine, *N* asparagine, *T* threonine, *Y* tyrosine, *Na* nalidixic acid, *PB* polymyxin B, *Str* streptomycin, *Sul* sulphamethaxazole, *Tet* tetracycline, *O129* Vibriostatic agent

The isolates were PCR positive for repeat in toxin (*rtxC*), and *rstR*^{El Tor} genes confirming the El Tor biotype. The RTX toxins represent a family of important virulence factors that have disseminated widely among Gram-negative bacteria. The *rtx* gene cluster in *V. cholerae* encodes the presumptive cytotoxin (*rtxA*), an acyltransferase (*rtxC*), and an associated ATP-binding cassette transporter system. PCR assays developed for *rtxA* or *rtxC* in *V. cholerae* can differentiate El Tor biotype from classical biotype among O1 serogroup. Thus, the presence of *rtxC* gene in all the isolates confirmed the El Tor biotype of strains.

In *rstR* typing, all the isolates were PCR positive for *rstR*^{El Tor} allele and negative for *rstR*^{Classical} except one isolate which was isolated from Hyderabad during cholera outbreak in 2009. This isolate carried both *rstR*^{El Tor} and *rstR*^{Classical} alleles [18]. The *rstR* alleles are believed to be biotype specific and are classified into *rstR*^{classical}, *rstR*^{El Tor}, and *rstR*^{Calcutta} for classical, El Tor, and O139 alleles, respectively [19].

In MAMA PCR, all the isolates gave amplification with primers for classical biotype. This PCR is *ctx* allele specific and determines the alteration in *ctxB* gene of *V. cholerae* strains [10]. Further, this was confirmed by *ctxB* nucleotide sequencing. Nucleotide sequence analysis of the *ctxB* genes of representative outbreak El Tor strains of *V. cholerae* O1 revealed that the strains harboured the *ctxB* sequences identical to that of the classical biotype strains. The deduced amino acid sequences of all representative El Tor O1 strains were aligned with the *ctxB* sequences of the reference strains N16961 (El Tor) and 569B (classical) (Table 1). The amino acid sequences of the isolates differed from the El Tor strain at position 39 (histidine in place of tyrosine) and at position 68 (threonine in place of isoleucine) and the sequences were similar to that of classical biotype strains (Table 1). Previously also, similar change in the amino acid sequences of CT has been reported from several countries [15, 20, 21]. However, in the isolates collected from Orissa outbreak in 2007, histidine at position 20, which was common in the prototype El Tor and classical strains, was replaced by asparagine indicating a further mutation in the gene (Table 1). Subsequent research conducted on these new mutants demonstrated the improved virulence compared to the prototype El Tor strains due to increase in toxin production [22]. Recently, *V. cholerae* isolates with similar mutation have been found in a major cholera outbreak in Haiti [4]. Genotypically, *V. cholerae* O1 biotypes are differentiated by *rstR* allele, the type of CT produced and presence of *rtxC* gene [9, 20]. Results of this study showed two genotypic evidences supporting El Tor biotype (*rtxC* and *rstR*), whereas *ctxB* gene sequence and MAMA PCR supported the classical biotype.

Classical and El Tor biotypes of *V. cholerae* O1 are closely related in their O-antigen biosynthetic genes. However, the

genomic structure of the CTXΦ, in which the CT genes are contained, differs between the classical and El Tor biotypes [14]. Among *V. cholerae* O1 strains, 3 types of *ctxB* genotypes have been identified based on amino acid residue substitution at 39, 46 and 68. All classical and US Gulf Coast El Tor strains have been grouped in genotype 1 or *ctxB1* on the basis of point mutations i.e. Histidine at 39, Phenylalanine at 46 and Threonine at 68, the Australian El Tor strains as genotype 2 or *ctxB2* i.e. Histidine at 39, Leucine at 46 and Threonine at 68 and El Tor strains of the seventh pandemic and Latin American epidemics as genotype 3 or *ctxB3* i.e. Tyrosine at 39, Phenylalanine at 46 and Isoleucine at 68 [2]. All the *V. cholerae* isolates from different outbreaks in India in this study belonged to genotype 1 i.e. harboured *ctxB1* allele except a few novel mutations in 2007 (Table 1). The results from this study confirmed the wide spread of the El Tor biotype with modified CT in Indian subcontinent indicating a continuous evolution of *V. cholerae* strains in the epidemic areas.

Antibiogram

Antibiotic susceptibility of all the outbreak isolates revealed the resistance for co-trimoxazole, nalidixic acid, polymyxin B, spectinomycin, streptomycin, sulfamethoxazole and trimethoprim (Table 1). However, the isolates were sensitive to ampicillin, cefixime, chloramphenicol, clindamycin, doxycycline, erythromycin, gentamicin, neomycin, norfloxacin, ofloxacin and rifampicin. The isolates were also resistant to the vibriostatic compound O/129, a phenotype reflecting trimethoprim resistant dihydrofolate reductase. *Vibrio cholerae* isolates before 2010 were sensitive to tetracycline. However, the isolates from a recent cholera outbreak in Orissa, Eastern India were found resistant to tetracycline. Tetracycline is an important broad spectrum antibiotic used for the prophylaxis and treatment of variety of bacterial infections. This is also a preferred choice among the antibiotics for control of cholera as recommended by WHO. Tetracycline, β-lactam, sulfamethoxazole/trimethoprim and quinolones cover most of the antimicrobials prescribed in India. Many cost effective antibiotics like co-trimoxazole and erythromycin have already become ineffective due to bacterial acquired resistance. Now, resistance for tetracycline further curtails the list of cost effective first-line drugs. Multi-drug resistance among *V. cholerae* strains limits the therapeutic potential of these drugs and presents additional challenges during disease management. Continued monitoring of antimicrobial susceptibility as well as strain-tracking are important for adapting policies for cholera control at national and global levels.

Thus, the study concluded that *V. cholerae* strains are continuously evolving as classical biotype strains of 6th pandemic were replaced by El Tor biotype in the seventh

cholera pandemic followed by the emergence of O139 and recently emergence of altered El Tor variants. Continuous emerging resistance for commonly used antibiotics demonstrates an evolutionary optimization of the organism, which could present a new and more significant emerging form of pathogenic *V. cholerae*.

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