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Research Article

Molecular Characterization of *Vibrio cholerae* Outbreak Isolates from Western India

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***Vibrio cholerae* isolates obtained from fecal samples referred from an outbreak of acute watery diarrhea in Rahude village in the Nashik district of Maharashtra, India, were characterized. The five culture-confirmed *Vibrio cholerae* isolates were subjected to molecular and phylogenetic analysis. The PCR and sequencing results of the *ctxB*, *rstA*, *rstB*, and *tcpA* genes confirmed that the Nashik *V.cholerae* strain belonged to the subtype O1 Haitian-type strain of the *ctxB*7 genotype. Moreover, the phylogenetic analysis of the *tcpA* gene suggested that the Nashik strain is closely related to the newly discovered polymyxin B-sensitive strain. This supports the recent finding on the prevalence of the polymyxin B-sensitive strain in India. The mutation in the *tcpA* protein was mapped to the $\alpha\beta$-loop region, which plays a role in pili formation as well as in the interaction of pili with the local environment.**

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Introduction

Acute diarrheal disease is a major public health concern in India and across the world [1][2]. *Vibrio cholerae* is the etiological agent of cholera, a devastating watery diarrheal disease. The occurrence of cholera has been divided into seven pandemics since 1817 [3][4][5]. In contrast to the previous pandemics, which were caused by the classical biotype, the 7th pandemic was caused by the less virulent El Tor biotype [6]. However, in the recent past, El Tor strains have become more virulent by acquiring genetic determinants of the classical strains [7].

The three genetic determinants that account for the virulence of *V. cholerae* strains include the bacteriophage CTXΦ-encoded core region and two *Vibrio* pathogenicity islands, VPI1 and VPI2 [8]. The core region of the CTXΦ phage contains *ctxA* and *ctxB* genes present in the O1/O139 strains and transcribes the potent A-B type enterotoxin *ctxA* and *ctxB* proteins. The ADP-ribosylation activity of ctxA is responsible for secretory diarrhea [9]. Another toxin gene, *rtxA* (repeat in toxin), is present in VPI2, which is also a marker for non-O1/non-O139 pathogenic strains of *V. cholerae* [10]. Other than the core region, the RS2 region contains *rstA*, *rstB*, and *rstR* genes that are involved in the replication, integration, and regulation of site-specific recombination of CTXΦ [11]. The VPI1 contains the *tcpA* gene, which encodes colonization factors—toxin-coregulated pili (TCP)—that mediate the attachment of bacteria to the intestinal epithelium [12][13].

Non-random genetic changes occur in the virulent genes of *V. cholerae*. These changes are systematically acquired in the *Vibrio* genome with a direct impact on the virulence of the bacteria. In 1990, an atypical El Tor variant with the classical cholera toxin gene started circulating in South Asia. With the passage of time, the atypical El Tor strain acquired new single nucleotide polymorphisms (SNPs). Based on these new SNPs, strains are divided into three waves, and the third wave containing strains like the ctxB-3b strain and the Haitian strain (ctxB-6) are known to cause severe cholera [7]. Thus, the sequence analysis of *V. cholerae* virulence genes and a comprehensive evaluation of genetic mutations are critical for understanding the phylodynamics of *V. cholerae* [14]. The present study reports the molecular characterization of culture-confirmed *V. cholerae* isolates obtained from an outbreak of cholera in July 2018. A brief summary of the outbreak is as follows: The cholera outbreak occurred in Rahude village, Nashik district, Maharashtra, between July 8 and 13, 2018, affecting a population of 850. A total of 195 cases were reported, yielding an attack rate of 22.9%. Two deaths occurred, resulting in a case fatality ratio of 1.03%. Most affected individuals were adults (72.8%), and women were disproportionately impacted. Poor sanitation, unsafe drinking water from a single open well, and heavy rainfall contributing to flooding and fecal contamination were critical risk factors that facilitated rapid transmission in this remote, low-

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socioeconomic community. The outbreak occurred in a remote tribal population in Rahude village of Nashik district, Maharashtra, a state located in western India[5][15]. In addition, phylogenetic analysis of the virulence genes (*ctxB, rstA*, *rstB*, *tcpA*, and *rtxA*) was also performed.

Materials and Methods

Bacterial Strains

V. cholerae

Five isolates of *V. cholerae* obtained from fecal samples referred from an outbreak of acute watery diarrhea at Rahude village, Nashik district, Maharashtra, in July 2018 were used in the study. The Institutional Ethics Committee was informed about the outbreak and approval was taken for conducting a post-outbreak investigation. The isolates were confirmed as *Vibrio cholerae* O1 Ogawa biotype El Tor using standard culture methods and slide agglutination with specific *Vibrio cholerae* polyvalent O1 antisera, followed by Ogawa and Inaba serotype-specific antisera (Denka Seiken, Japan) as well as phenotypic tests for biotype differentiation (hemolysis, Chick RBC agglutination, and Voges-Proskauer reaction) [16]. The isolates plated on non-inhibitory media (Trypticase Soy Agar-TSA) were further suspended in phosphate-buffered saline (PBS) and used for the extraction of genomic DNA as described below.

Diarrheagenic E. coli

Three to six lactose-fermenting colonies and up to three non-lactose-fermenting colonies from MacConkey (MAC) agar plates were selected for testing by conventional procedures [16]. Identification of *E. coli* isolates was confirmed by the VITEK® 2 COMPACT automated microbial identification system (bioMérieux, Inc. Hazelwood, USA) and VITEK 2 GN cards (bioMérieux, Marcy-l'Ètoile, France). These isolates were replated on TSA, from which three isolated colonies were resuspended in PBS for further extraction of genomic DNA.

Polymerase chain reaction (PCR) assay and Phylogenetic analysis

The genomic DNA was extracted from the bacterial isolates using the QIAamp DNA kit (Qiagen, USA), and singleplex PCRs were performed for specified targets using the AccuPrime *Taq* DNA Polymerase System (Thermo Fisher Scientific). The specific primers, annealing temperatures, and expected amplicon sizes for each gene target are detailed in Table 1. To confirm the identity of the PCR products, both strands of the amplified products were sequenced using the Big-Dye terminator cycle sequencing kit (Applied Biosystems) followed by sequencing in an automated nucleotide sequencer (Bioanalyzer 3100; Applied Biosystems).

Sr. No	Gene Target	Forward Primer	Reverse Primer	Annealing Temp (°C)	Amplicon Size (bp)	Reference
1.	*ctxA*	CTC AGA CGG GAT TTG TTA	TCT ATC TCT GTA GCCCCT	54	301	[17]
2.	*ctxB*	GGT TGC TTC TCA TCA TCG AAC CAC	GAT ACA CAT AAT AGA ATT AAG GAT	54	460	[17]
3.	*rstA*	CCACGT GT AGA GCA	GAG TGA ATC GTC GTG	55	539	[18]
4.	*rstB* (with GTA)	CTC ATT CTG AAG GGG TGA GTA A	GGT GCA CCA GTC TTA CAA	50	372	[19]
5.	*rstB* (with GTA deletion)	CTC ATT CTG AAG GGG TGA GTA A	GCA CCA GTC TTA CGT AC	50	372	[19]
6.	*tcpA*	GGT GGG CAT AGT GAT AAG AG	CGC CTC CAA TAA TCC GAC AC	55	1050	[20]
7.	*gyrA* (For El Tor Variant)	TGC TCT TCC TGA TGT GCG TGA TG	GAT GGT GTC GTA AAC CGC **TA**	60	177	[21]
8.	*gyrA* (For El Tor)	TGC TCT TCC TGA TGT GCG TGA TG	GAT GGT GTC GTA AAC CGC **TC**	61	177	[21]
9.	*rstR*	GCA CCA TGA TTT AAG ATG GTC	TCG AGT TAA TTC ATC AAG AGT G	55.2	411	[22]
10.	*rtxA* (For El Tor Variant)	ATC GGA ATG AGT GAG AAA GAC C	TGT GAA CCA CGT CTG **CT**	54	187	[21]
11.	*rtxA* (For El Tor)	ATC GGA ATG AGT GAG AAA GAC C	TGT GAA CCA CGT CTG **CC**	54	187	[21]
12.	*rtxA* (For full gene sequencing)	TAC TTT AAT GGT AAC CGC GCT	CAT TGT CAC TGT ACT TAC GTC	54	422	[21]

Table 1. Primer sequences, amplicon size, and annealing temperature used in the PCR assays

The phylogenetic analysis of the *V. cholerae* isolates from the Nashik outbreak was performed for the *ctxB*, *rstA*, *rstB*, and *tcpA* genes. The sequencing results were confirmed by an alignment search using BLAST [23]. The BLAST hits mentioned in the literature were selected, and their sequences were retrieved for further analysis from the public database NCBI [24]. The *ctxB* and *rstA* gene sequences were aligned with the corresponding gene sequences of atypical El Tor strains [7]. The phylogenetic analysis for the *tcpA* gene was performed by comparing it with *tcpA* gene sequences from toxigenic and non-toxigenic isolates as reported by Kumar et al. (2011). In addition, the *tcpA* sequences from classical strains (O395), the El Tor strain (MJ 1236), the ZJ59 strain, and the WB-0050 polymyxin B-sensitive strain were also included [25][26].

The multiple sequence alignment was performed using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) tool available in the Molecular Evolutionary Genetic Analysis program version 7 (MEGA), and gap regions were removed. The phylogenetic tree of the trimmed alignment was prepared using the MEGA program's Neighbor-joining method with 2000 bootstraps [27][28].

Mapping SNP on tcpA protein structure

Sequence alignment was performed for the tcpA protein sequences of the Nashik strain and the El Tor strain. The coordinates of a single chain (chain A) of $tcpA^{cla}$ from the classical strain (PDB ID 1OQV) and $tcpA^{El}$ from the El Tor strain (PDB ID 3HRV) were obtained from the PDB database, and structure-based sequence alignment was performed using $tcpA^{El}$ as a template [29][30]. The mutated amino acid was mapped on $tcpA^{El}$ using UCSF Chimera software [31]. In addition, the αβ-loop region and D-region were mapped on the $tcpA^{El}$ structure. The residues that are different in $tcpA^{El}$ and $tcpA^{cla}$ were also mapped [32]. The solvent accessibility of $tcpA^{El}$ was determined by ASAVIEW [33].

Results and Discussion

Progressive changes in the genomic sequence of *V. cholerae,* in the form of SNPs, intra-strand, and inter-strand chromosomal recombination are known, which lead to novel and diverse strains. The virulence regions like the core region, RS2 region, and *Vibrio* pathogenicity islands VPI1 and VPI2 gain such non-random genetic variations [34]. This study has reconfirmed *V. cholerae* as the main etiological agent of acute diarrhea in Rahude village in Nashik. The Nashik strain was found positive for virulence genes of all three virulence regions (*ctxA*, *rtxA*, *ctxB*, *rstA, rstB*, and *tcpA*), suggesting the presence and intactness of both the core region and *Vibrio* pathogenicity islands. The presence of *ctxA* and *ctxB* confirmed the presence of the main CT antigens. The sequencing comparison of virulence genes among different isolates implicated complete identity, suggesting the prevalence of only a single strain of *Vibrio* in Rahude village.

Consequently, molecular characterization of *ctxB*, *rstA, rstB*, and *tcpA* was performed [35]. The SNPs in the ctxB gene sequence serve as the basis for differentiating classical and El Tor biotypes and CTB epityping [36]. The presence of SNP A58 (a change in amino acid residue 20) on *ctxB* suggested that the Nashik 2018 strain belongs to the O1 *ctxB*7 genotype. The results of the phylogenetic analysis of the Nashik strain *ctxB* gene sequences show that atypical El Tor strains diverged from El Tor strains N16961 and India 1991. The phylogenetic tree of *ctxB* genes showed that the atypical El Tor strains are distributed into two clusters. The first cluster consisted of wave 1 and wave 2 strains. The third wave, which includes ctxB-3b, the Haitian strain (ctxB-6), and the newly reported polymyxin B-sensitive strain, is included in the second cluster (Figure 1) [14][37]. The Nashik strain was also found to cluster in the third wave. The SNP mapping of *ctxB* suggested a similarity between the Nashik strain, the Haitian-type strain (also categorized as CTX-6b), and the CTX-3b type. Both the Haitian strain and CTX-3b are highly virulent strains responsible for severe cholera outbreaks. The CTX-3b emerged in 1996 in India and is gradually replacing the CTX-3 strain in the Indian subcontinent [38]. The difference between the Haitian and CTX-3b strains was determined by sequencing the *rstB* gene. The absence of the trinucleotide 'GTA' at positions 74-76 nt in *rstB* implicated that the Nashik strain is a Haitian-type strain [7]. This result is in congruence with the finding that the *ctxB7* Haitian strain is presently in circulation in India [39].

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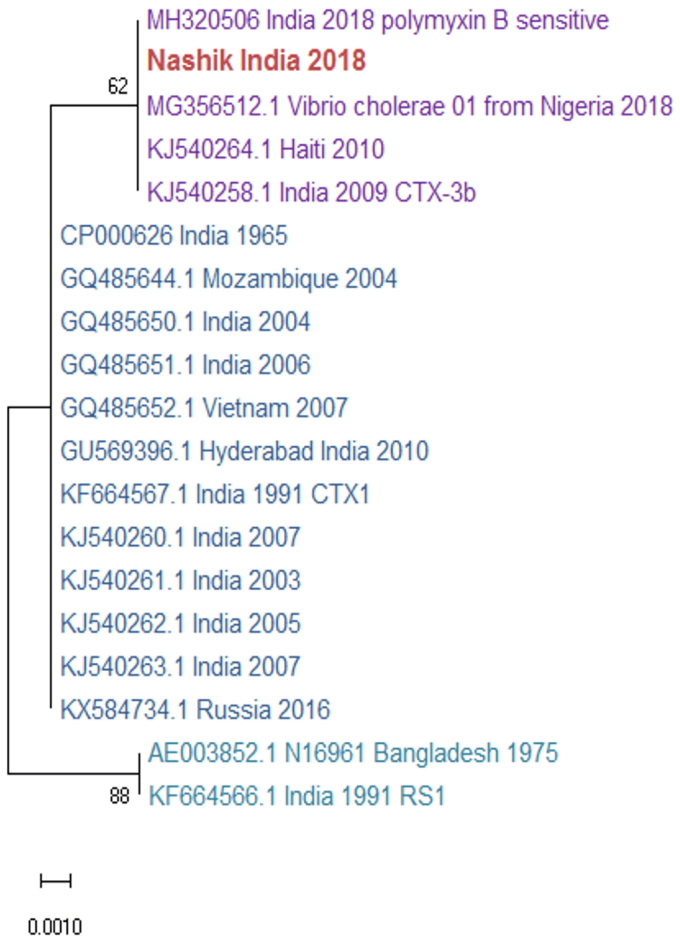

Figure 1. The phylogenetic tree of the *ctxB* gene constructed using the neighbor-joining method as implemented in MEGA. Bootstrap values are the percentage of 2000 replications. The first and second waves are in blue, and the third wave is in violet. The Nashik strain is shown in red.

The molecular analysis of the partial sequence of the *rstA* (from 435 to 1083 nucleotides) gene revealed the presence of C927, T933, and T942. The *rstA* phylogenetic analysis grouped all atypical El Tor strains, except V212-1, V212-2, the CTX-2 Mozambique 2004 strain, and the CTX-3b India 2006 strain. The Nashik strain also clustered in the atypical El Tor strains cluster (Figure 2). Thus, the analysis of *ctxB* and *rstA* reinforces that the Nashik 2018 strain is part of the third wave of atypical El Tor strains, clustering with the Haitian strain, the CTX-3b strain, and the polymyxin B-sensitive strain [7][26]. The PCR analysis of the *rstB* gene detected the presence of the trinucleotide GTA at nucleotide positions 74-76 [37], indicating that the Nashik strain is a Haitian-type strain.

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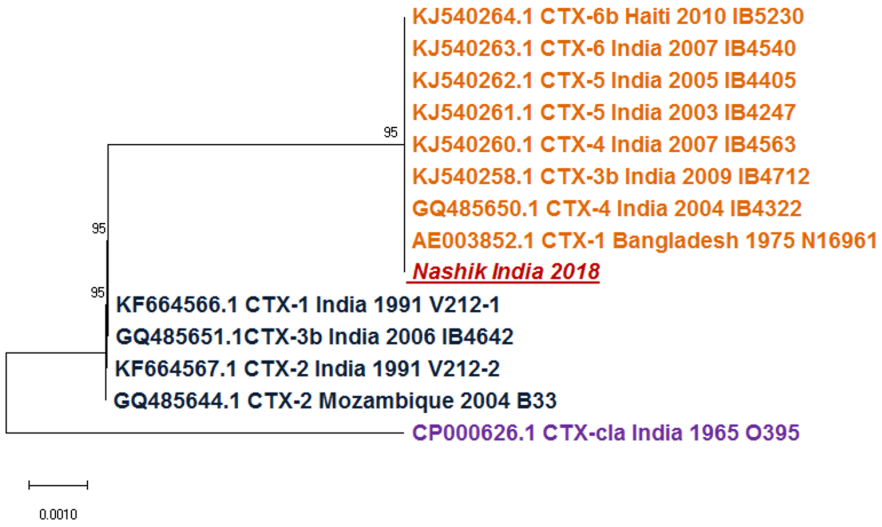

Figure 2. The phylogenetic tree of *rstA* constructed by using the neighbor-joining method as implemented in MEGA. Bootstrap values are the percentage of 2000 replications. The Nashik strain is colored red, italicized, and underlined.

The phylogenetic tree of the *tcpA* gene revealed the distribution of *Vibrio* strains into 10 phylogenetically divided clusters as reported by Kumar et al. [40]. The Nashik strain *tcpA* gene was part of cluster number 9, along with the MJ-1236 strain (an El Tor strain). However, the Nashik strain *tcpA gene* showed more sequence identity to the *tcpA* gene from the polymyxin B-sensitive strain, thus originating a new subcluster (Figure 3). The polymyxin B-sensitive strain in India has been documented since 2016, with circulations of both polymyxin B-resistant and -sensitive type strains recently reported from Maharashtra and Gujarat [26]. Polymyxin B sensitivity is one of the consistent phenotypic traits of the classical strain, which is now being acquired by the El Tor biotype. Thus, the isolation of a polymyxin-resistant strain from Rahude village, a tribal area on the border of Maharashtra and Gujarat, is of significance.

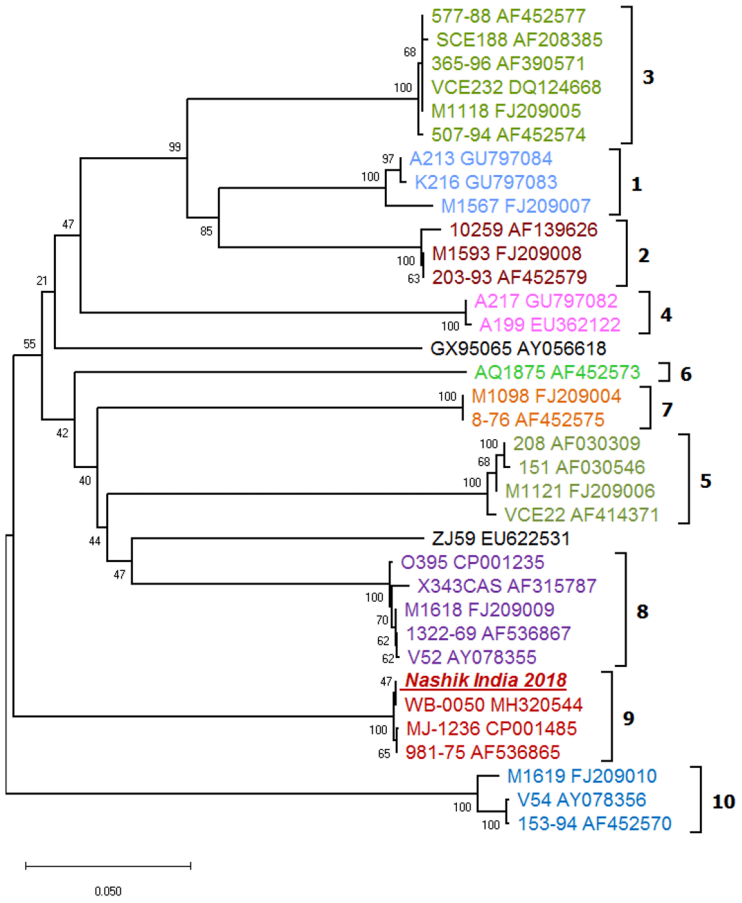

Figure 3. The phylogenetic tree of the *tcpA* gene constructed using the neighbor-joining method as implemented in MEGA. Bootstrap values are the percentage of 2000 replications. The phylogenetic tree is categorized into 10 clusters. The Nashik strain is in red font, italicized, and underlined.

The presence of SNP A266G in the *tcpA* of the Nashik and polymyxin B-sensitive strains leads to a mutation at the 64th position of the tcpA protein from asparagine to serine (N64S). The N64S mutation is mapped to the αβ-loop region, which plays a role in pili formation as well as interaction with the local environment [41][42]. The tcpA protein belongs to the class of type IV pilin proteins, and the αβ-loop region shows high structural as well as sequence variability among type IV pilin proteins from different species and also among different biotypes of *V. cholerae*. The functional relevance of the N64S mutation is still unknown [39][43]. However, it has been reported that the N64S mutation does not affect the virulence of *V. cholerae* [44]. The change from threonine to asparagine and then to serine occurred at the 64th position in the classical, El Tor, and polymyxin-sensitive strains, respectively, indicating a regain of the hydroxyl (-OH) group. Notably, tcpA not only plays a role in adherence but also is critical in CTXΦ phage invasion of the *Vibrio* bacterium [45]. The amino acid at the 64th position has a high solvent-accessible area (as determined by ASAVIEW, data not shown) that might indicate a functional role for the N64 residue [33].

In addition, two *E.coli* isolates from fecal samples negative for *V.cholerae* were found to be positive for the EPEC virulence *eae* (intimin) gene, a marker for the pathogenic *E. coli* EPEC strain. However, these isolates were negative for another EPEC gene, *bfpA* (bundle-forming pili). The presence of (*eae*+, *bfpA*-) strains is suggestive of an atypical EPEC strain [46]. The molecular detection of atypical EPEC in two *Vibrio*-negative isolates (sample nos. 52 and 78) indicates more than one microbial etiology in the diarrheal outbreak. The target *bfpA* and *eae* genes are crucial for the three-stage model of EPEC infection [47]. Non-intimate attachment is formed by *bfpA* in the first stage, whereas in the third stage, the intimin protein (coded by the *eae* gene) leads to intimate attachment [47]. Deletion of the *eae* gene reduces the pathogenicity of EPEC, whereas no significant effects occur in the absence of the *bfpA* gene [48]. The positivity of atypical EPEC

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(*eae*+, *bfpA*-) during a cholera outbreak is intriguing, as *bfpA* and *tcpA* both belong to the same class of type IVb pilins [47].

Limitations

A limitation of this study is the small number of isolates (n=5). While this is sufficient for characterizing a single clonal outbreak, it limits the generalizability of our findings to the broader *Vibrio cholerae* population in the region.

Conclusion

This study highlights the molecular and phylogenetic characterization of *V. cholerae* strains isolated during the 2018 Nashik outbreak. The information obtained shows that it is an altered El Tor O1 *ctxB*7 genotype Haitian-type strain, with genotypic similarity to a polymyxin B-sensitive strain. The mutation present in the TcpA protein of the Nashik strain and the polymyxin B-sensitive strain was mapped to the αβ-loop region, suggesting a probable effect on pili formation or the interaction of pili with the local environment. In addition, atypical EPEC lacking the *bfpA* gene was also detected. Taken together, the findings support the molecular characterization of the genetic makeup of the *Vibrio* strain and its virulence, along with providing evidence for more than one microbial etiology. In conclusion, this study reinforces the need for continuous molecular surveillance of diarrheal outbreaks.

Statements and Declarations

Funding

No specific funding was received for this work.

Potential Competing Interests

The authors declare no potential competing interests.

Ethics

This study was approved by the ICMR-NIV ethics committee. The requirement for written informed consent was waived by the ICMR-NIV ethics committee because the study was part of a public health surveillance response and utilized de-identified samples. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Availability

The sequence data analyzed in this study are available in the public NCBI database (NCBI IDs: MK873008 (ctxB), MK873009 (rstA), MK873010 (rstB), and MK873011 (tcpA)). Additional data may be available from the corresponding author upon reasonable request.

Author Contributions

Anuj Kumar performed the molecular analyses and drafted the manuscript. Rajlakshmi Viswanathan contributed to sample collection, bacterial culture, and manuscript revision. All authors read and approved the final manuscript.

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