RESEARCH ARTICLE

Evaluation of *Vibrio cholerae* O1 El Tor variants circulating in Sindh Pakistan

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Abstract

Objective: To investigate the existence of genetically diverse vibrio cholerae variant strains in a rural Sindh district, and to find out the phylogenetic relationship of indigenous vibrio cholerae strains.

Methods: The cross-sectional study was conducted from April 2014 to May 2016 in Khairpur, Pakistan, and comprised stool samples/rectal swabs collected from the main and city branches of the Khairpur Medical College Teaching Hospital, and the Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences, Gambat. The samples were identified using standard microbiological, biochemical, serological techniques and polymerase chain reaction targeting the ompW gene. Whole genome sequencing and bioinformatics tool MUMmer 3.2.3 was used to compare indigenous and contemporary vibrio cholerae strains circulating in the province of Sindh. Neighbour-joining tree method was used to construct the phylogenic tree.

Results: Of the 360 samples, 76(21.11%) were found positive for vibrio cholera strains. The species-specific ompW gene was amplified at the correct size of 588bp. The isolates belonged to serogroup Inaba, O1, biotype El Tor. Unique sequences with same genomic coordinates showed that test strains were not similar to the reference sequence. Conserved genome sequences showed that 12 Out of 16 (75%) of the test strains were similar to each Other except the 3 strains isolated from Khairpur and 1 from Karachi. Multiple sequence alignment of the regions translated into protein showed that 13 out of 16 (81.25%) test strains were similar except 2 strains from Khairpur and 1 From Karachi. The phylogenetic tree showed that all isolated strains descended from the same ancestor along with the reference strain.

Conclusions: Vibrio cholerae O1 El Tor variant existed in Khairpur.

Keywords: Cholera, Vibrio cholerae, Variant, Khairpur, Phylogeny, MUMmer. (JPMA 72: 2381; 2022)

DOI: https://doi.org/10.47391/JPMA.1942

Introduction

Cholera is an acute human disease characterised by watery diarrhoea and vomiting. Aetiological agent is a filamentous, comma-shaped bacterium named vibrio (V.) cholerae. So far, seven pandemics have been reported worldwide since 1817 where cholera remained a pandemic for 1000 years in South Asia.[1-3] On the basis of phenotype and genotype, V. cholera has been classified into V. cholerae biotype eltor (El Tor). The first six pandemics were caused by the classical biotype, but in 1905, El Tor appeared to be the predominant biotype responsible for the seventh cholera pandemic in 1961.[4,5] After a few years, serogroup O139 was replaced by O1 serogroup and now it rarely appears.[6] Epidemic of O139 was observed 6 years after the 1993-1994 epidemic in India and in developing countries. A recent outbreak of cholera has been reported in 2017 from Yemen that claimed 332, 658 cases and 1759 deaths.[7]

In the environment, water reservoirs and brackish water are the main reservoirs for V. cholerae. Cholera still persists as endemic due to poor hygienic conditions and unsafe drinking water, infected households and environments where V. cholerae exist naturally.[8] During endemics, even healthcare workers acquire infection due to person-to-person contact.[9] Pathogenicity of V. cholerae is a result of protein toxins, such as cholera toxin, responsible for severe diarrhoea with high fatality rate.[10]

In 2010, there was a surge in cholera cases due to severe flooding that threatened public health across Pakistan, with 164 laboratory-confirmed cases.[11] Cholera remains a major health problem in Pakistan, including the province of Sindh, and is responsible for many cases each year, especially in the northern areas.[12] In the recent past, variant El Tor V. cholera cases have been reported from different countries, but no relevant data is available from rural Sindh. The current study was planned to investigate the existence of genetically diverse V. cholera variant strains in a rural Sindh district, and to find out the phylogenetic relationship of indigenous V. cholerae strains.

Materials and Methods

The cross-sectional study was conducted from April 2014 to May 2016 in Khairpur, Pakistan, and comprised stool samples/rectal swabs collected from the main and city branches of the Khairpur Medical College Teaching Hospital, and the Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences, Gambat, Pakistan. The samples were collected using simple random sampling technique and the sample size

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calculation was done using online sample size calculator.[13] The patients included were either gender aged 05-60 years who had watery diarrhoea cholera or bloody diarrhoea dysentery, had onset of illness less than 4 days before sampling, and who had not received antimicrobial treatment for the diarrhoeal illness. Those not meeting the criteria were excluded. After taking informed consent from the patients, or their parents/guardians, data was collected using predesigned data forms. Strains from Khairpur were designated as Amjad Mughal V. cholera (AMVC), those from Karachi as Karachi Civil Hospital (KCH), and those from Jamshoro as Kotri Talka Hospital (KTH).

All the collected samples were transferred to the Molecular Biology Laboratory, Institute of Microbiology, Shah Abdul Latif University (SALU), Khairpur, for enrichment in alkaline peptone water.[14] (APW). The enriched samples were incubated aerobically at 35°C for 24h.

On the following day, the enriched samples were cultured aerobically at 35°C for 24 hours on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar media (Oxoid).

Growth was observed for distinct yellow V. cholerae colonies. These colonies were gram-stained and sub-cultured on non-selective nutrient agar (Oxoid) and incubated aerobically at 35°C for 24 hours. Standard microbiological techniques, such as gram-staining and motility test using hanging drop technique were performed. Isolated colonies from the nutrient agar were subjected to analytical profile index (API) 20E (enterobacteriaceae), as per manufacturers' recommendations.

String test was performed for the confirmation of the isolates as V. cholera by the formation of string while pulling the emulsified culture on a slide with the help of sterile wire loop. The isolates were tested for biotype, using conventional Voges-Proskauer (VP) test and antibiotic sensitivity against polymyxin B (Oxoid 50U disc). V. cholerae classical was sensitive, while El Tor was resistant to polymyxin B.

Polyvalent V. cholera O1 and O139 anti-sera, and monovalent anti-sera against Inaba and Ogawa (Denka Seiken Co. Japan) were used by slide agglutination at room temperature. V. cholerae O1 were kept frozen at -20°C in glycerol for future use. Species-specific polymerase chain reaction (PCR) was performed targeting ompW gene (588bp) using specific primers.[15] V. cholerae ATCC 14035 was used as positive control. The deoxyribonucleic acid (DNA) was extracted by boiling method and the PCR was performed.[16] The amplified DNA products were visualised using 1% agarose gel electrophoresis under an ultraviolet (UV) transilluminator (BioRad, United States).

Three representative indigenous isolates of V. cholerae, AMVC 58, AMVC 64 and AMVC 256, from Khairpur were selected for comparative genomic study with V. cholerae El Tor strain N16961 (Accession No. AE003852-3),[17] alongside 5 KTH and 8 KCH strains. Genomic DNA of all strains was sequenced using Illumina HiSeq Platform (Sangar, USA). The soft data of whole genomes were obtained in Fast-All (FASTA) files for further analysis at the Genomics Laboratory, Department of Biomedical Engineering, Latif Ebrahim Jamal (LEJ) campus, Nadirshaw Edulji Dinshaw (NED) University, Karachi.

The diverse and similar regions among the genomes were identified using maximal unique match (MUMmer), nucleotide match (NUCmer) and protein match (PROmer).[16] The phylogeny of diverse regions was identified amongst the strains. The region of reference strain was selected that has shown similarity with other strains. PROmer was used to align the translated DNA into amino acids. The selected reference genome sequence of V. cholerae regions and most similar regions from all other strains under study were then subjected to Clustal Omega European molecular biology laboratory (EMBL) software for generating multiple sequence alignment (MSA) and phylogeny. The phylogenetic inference was based on the neighbour-joining (NJ) distance method.[18] For MUMmer and NUCmer, all strains contigs were analysed with the reference strain in order to find out the similar regions among the sequences. The most interesting region from all strains having same position/coordinates was then selected along with the reference sequence for creating new FASTA file. This file was then uploaded to Clustal Omega software for generating MSA. In case of PROmer, all the regions were translated using National Centre for Biotechnology Information (NCBI) open reading frame (ORF) finder before MSA.

Results

Of the 360 samples, 76(21.11%) were found positive for V. cholera strains. On TCBS agar, typical sucrose fermenting yellow shiny colonies (2-4mm) were found (Figure-1A), while on the on nutrient agar, translucent shiny colonies were observed (Figure-1B).

Gram-staining showed gram-negative curved rods and darting motility. The optimum time for growth was 24h and optimum temperature was 35°C for the isolated V. cholerae.

Agglutination of the indigenous strains with polyvalent anti-sera indicated that the V. cholerae belonged to serogroup Inaba, O1. The isolated strains were found VP+ and polymyxin-B resistant which confirmed the El Tor biotype. The biotype classification of the V. cholerae O1 strains revealed typical El Tor phenotype similar to the reference strain El Tor N16961. API 20E profiling showed POSITIVE REACTION on O-nitrophenyl-beta-D-galactopyranoside (ONPG), lysine decarboxylase (LDC)), ornithine decarboxylase (ODC), citrate (CIT), indole (IND), Voges-Proskauer (VP), gelatinase (GEL), glucose (GLU), mannose (MAN), sucrose (SAC). NEGATIVE

Table: Phenotypic characters of vibrio (V.) cholera isolated from the clinical samples.

	Site	Total samples (n)		Phenotype	Genotype ompW gene
	Khairpur	320	+	Yellow colonies on TCBS, G+, motile, IMViC +	Present (588 bp)
Sero-group				01 Inaba	
Bio-type				El Tor	
	Gambat	40	+	Do	do
Grand Total		360			

TCBS: Thiosulfate-citrate-bile salts-sucrose, IMViC: Indol, Methyl red, Voges- Proskauer Citrate.

REACTIONS were showed in arginine dihydrolase. (ADH), hydrogen sulfide (H2S), urea (URE), tryotophane deaminase (TDA), sorbitol (SOR), rhamnose (RHA), melibiose (MEL) and amygdalin (AMY). The reference API ID 5347124 matched for V. cholera tested. Agarose gel electrophoresis revealed successful amplification of ompW gene in all the clinical isolates at correct position of 588bp, comparable to the V. cholerae strain ATCC 14035 used as positive control in PCR (Figure-2).

MSA of the regions showed that the reference sequence was not similar to the tested strains where 8 out of 16 (50%) did not show sequences. Partial similarity (6 out of 16, 37.5%) was found except a few regions of KCH2, KCH9, KCH17, KCH18 AMVC64 AND KTH2.

The reference strain shared ancestor relationship with KCH-17. All KTH and KCH strains did not cluster possibly because the phylogenetic tree was constructed from unique sequences (Figure-3).

NUCmer identified conserved regions among all strains with different genomic coordinates, belonging to different regions. MSA from conserved regions showed that most of the sequences were conserved except sequences of KCH-10, KCH-15, AMVC-256, AMVC-58 and AMVC-64. Phylogenetic tree from NUCmer sequences showed that 5 out of 8 (62.5%) KCH sequences were placed together except KCH-10, KCH-15 and KCH-18 where KTH-4, KTH-6, AMVC-58 and AMVC-64 were placed together. AMVC-256 and strain KCH-10 had almost same root with AMVC-64, showing that all the three AMVCs had descended from the same ancestor, including KCH-10 and the reference strain sequence.

Similar regions of reference strain and other strains were then retrieved. These similar regions were found to be conserved. Therefore, they can be considered functional parts of the genome.

Out of 8 KCH strains, 7 were placed together in the tree except KCH-10. Possibly it was separated from its group because of mutations and migration. Alignment of selected regions translated into protein exhibited that proteins evolved slowly because of codon biasness. Therefore, these protein regions were more conserved compared to nucleotide sequences of the same region.

MSA of the regions translated into protein gave better understanding of evolution. The protein alignment showed that 7 out of 8 (87.5%) KCH strains, AMVC 256 and all the 5 KTH strains were similar except KCH-16 and AMVC-64. This divergence in two strains was possibly due to evolution. It was also observed that two amino acids were deleted in lower segment of the alignment in all the similar sequences which showed conserved nature of the sequences. Overall, MSA of unique sequences showed few regions of similarity where phylogenetic relationship showed distinct clades. Similar regions with different genomic coordinates showed

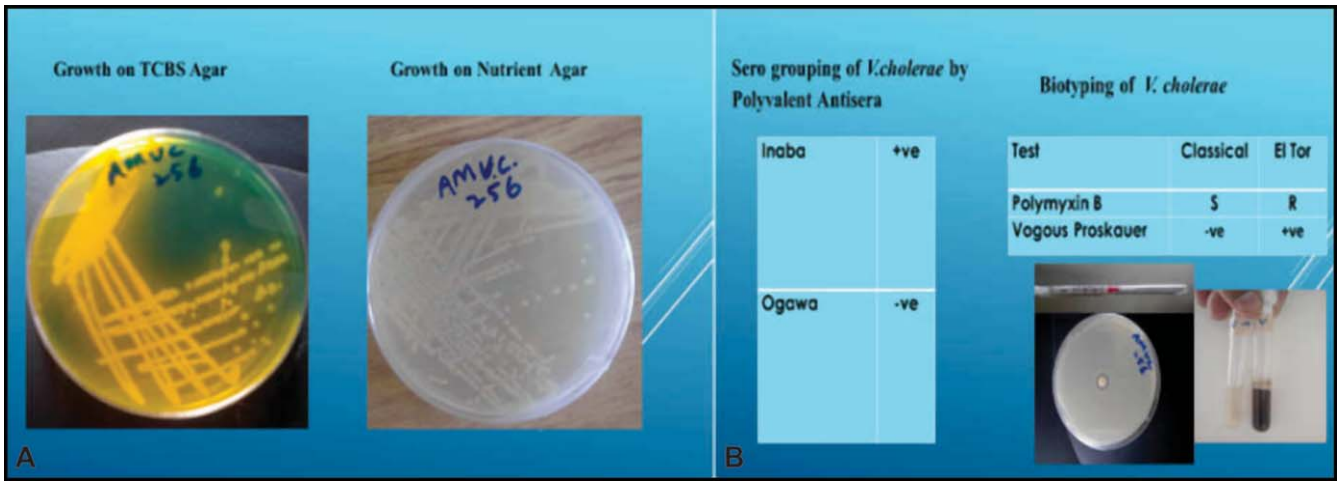

Figure-1: (A) Growth of vibrio (V.) cholerae from clinical samples. Left panel showing growth on thiosulfate-citrate-bile salts-sucrose (TCBS), and the right plate showing growth on nutrient agar. (B) Sero-grouping and bio-typing of clinical isolates of V. cholera. Left panel showing agglutination test positive (+) and negative (-) with polyvalent antisera, and the right panel showing the disk diffusion test showing resistance against polymyxin B. The tubes show positive and negative Voges-Proskauer (VP) test.

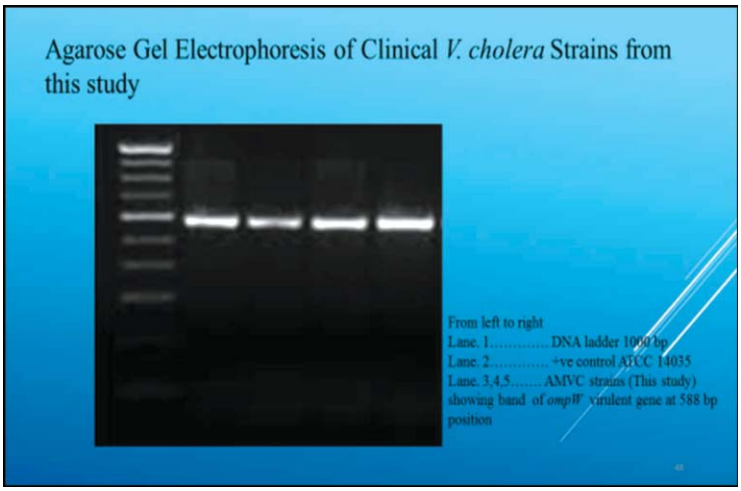

Figure-2: Agarose gel electrophoresis of polymerase chain reaction (PCR) - amplified ompW gene.

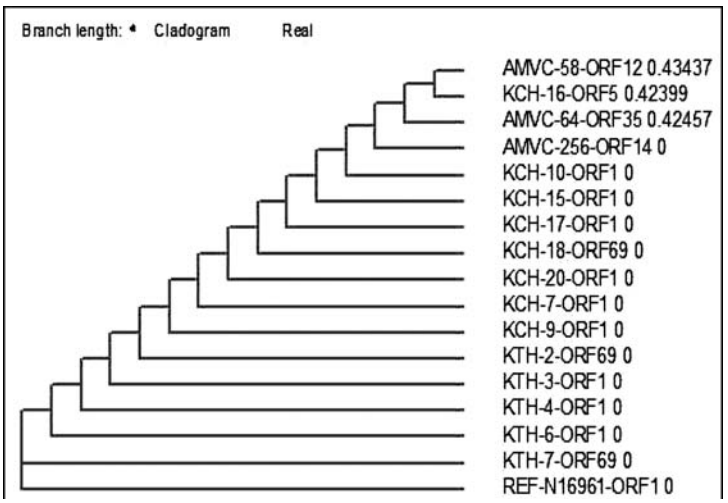

Figure-3: Phylogenetic tree of protein sequences from V. cholerae using protein match PROmer software.

that 13 out of 16 (81.25%) sequences were conserved except sequences from the Khairpur strains that probably changed during the process of evolution as well as KCH 16 strain acquired distinct linage. Translated protein sequence of reference strain did not share roots directly with the test strains, hence, all KCH strains were placed together except KCH-16 that clustered with AMVC strains where KTH strains showed similarity to each other, indicating slow protein evolution of each strain.

Discussion

To the best of our knowledge, the current study is the first to report the presence of V. cholera El Tor variants in Sindh, Pakistan.

Clinical strains of V. cholerae showed serogroup O1 Inaba which is in agreement with an earlier Pakistani study.[19] The isolates were confirmed as biotype El Tor, a phenotype comparable to 7th cholera pandemic strain V. cholerae N16961, which was used as reference strain in the current study, and disagrees with previous finding from Pakistan regarding the biotype classification in which V. cholera O139 was reported as the cause of cholera.[20]

Comparative genomic analysis of 16 strains isolated from Khairpur, Karachi and Jamshoro showed no similarity except a few regions of some strains, which indicate divergence among the sequences. Phylogenetic relationship among the strains substantiated this presupposition of diversity analysed through sequencing of genomes and uncovered variance among the strains. These sequences were changed probably during the process of evolution. Protein analysis is important in determining evolutionary patterns. PROmer translated nucleotide sequences into proteins of regions of interest from V. cholerae strains and their comparative analysis showed greater tendency towards being conserved compared to NUCmer alignment. It can be attributed to the slow evolving nature of protein molecules, since changes in nucleotide not necessarily cause change in amino acid. In protein alignment, most of the strains were found to be homologous to some extent,

Sharing the same ancestral root while showing distinct phylogenetic clades proposes a diverse relationship of strains isolated from the study region with contemporary strains, and indicates that cholera in Khairpur was caused by variant V. cholerae El Tor strains. The current results are in agreement with published reports.[11] It has been reported that clinical isolate of V. cholera from a patient in the United States who visited Pakistan, was a variant strain with deletion of VP1.[21,22]

Similar reports about El Tor variants has already been published from Haiti, Asia Africa and Bangladesh and Mexico.[23] The phylogenetically diverse genome observed in the current study may have been due to gene recombination and/or mutation in indigenous El Tor strains that resulted in El Tor variants. Consistent to these findings, El Tor variants in clinical isolates have also been reported from Bangladesh and Haiti.[24,25]

Recently, a study on comparative core genome analysis of V. cholera from Pakistan has been published where capability of genome modification by V. cholera has been highlighted.[26] These findings corroborate the current findings about El Tor isolates. It has been described in literature that the variants of El Tor biotypes showed enhanced symptoms of cholera that may cause increased death rate.[27] Further investigations are required at the gene level to substantiate the findings of the current study.

Conclusion

V. cholerae O1 El Tor variant existed in the area studied. There is a need for detailed phylogenetic studies and more sequence data, especially from the set of genes in the study area.

Limitations of the Study

The sample collection, identification and preservation of V. cholera was carried out during the period of 2014 -2016 and processed in Shah Abdul Latif University Khairpur whereas molecular characterization and sequencing was conducted in COMSATS Islamabad and further in NED University Karachi. The whole arrangement of the research was delayed due to completion of work in multiple institutions because of unavailability of required facilities in the Shah Abdul University Khairpur. The second reason of delay was long waiting time from the journal due to que of already submitted articles as communicated to the authors.

Disclaimer: The text is based on an academic thesis.

Conflict of Interest: None.

Source of Funding: Partially funded by Shah Abdul Latif University, Khairpur, Pakistan.

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