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A Comparison of Genetic Sequence of the *Vibrio cholerae* Strains Isolated from Iraq and Genetic Sequence of the *Vibrio cholerae* Strains Recorded Globally Based on *rec*A gene.

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ABSTRACT

This study were conducted in order to address the genetic relatedness between *Vibrio cholerae* isolated from Iraq and other countries based on housekeeping gene *rec*A sequence analysis. Here, the multiple sequence alignment analysis and neighbor joining phylogenetic tree analysis was performed by using Un weight pair group method with Arithmetic Mean (UPGMA tree) in MEGA 6.0 version that analyzes 562 pb region of the *rec*A from 20 *Vibrio cholerae* isolates obtained from human in Iraq, and compared with the sequence data from the isolates belonging to other place. The phylogenetic tree analysis that used for confirmative detection analytic results revealed the close relation of all local isolates of *Vibrio cholerae* to NCBI-Blast *Vibrio cholerae rec*A gene (U10162.1), whereas other NCBI-Blast pathogenic *Vibrio. speciesrec*A gene were out of tree at total genetic change (0.10-0.02%) and the phylogenetic tree analysis that used for genetic diversity analytic results revealed that all local *Vibrio. cholerae* isolates were more different than NCBI – Blast *Vibrio cholerae* isolates at total genetic change (8-2%). The present study represents the first report on the use of molecular phylogeny to comparative sequence analysis of *rec*A gene among *Vibrio cholerae* isolates from Iraq with globally reported sequences.

Keywords: *Vibriocholerae*, phylogenetictree, *rec*A, UPGMAtree .

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INTRODUCTION

Vibrio cholera is still a major public health concern in the developing countries (Banerjee*et al*,2014). It is the causative agent which can cause a considerable difficulties like diarrhea, dehydration, acidosis, shock and ultimately death(Ritchie&Waldor,2009). It remains a major cause of morbidity and mortality throughout world (Ratnam*et al*,2015). *Vibrio cholerae*is an important food borne pathogen worldwide, and it is endemic in developing and less developed countries that lack clean water supplies and public health facilities (Alam *etal*,2006). Vibrios are abundant worldwide in aquatic environments, including estuaries, marine coastal water and sediments, and aquaculture settings (Heidelbery *et al*,2002a;Suantika *et al*,2001;Urakawa *et al*,2000). Therefore, water plays a significant role in the transmission and epidemiology of cholera (Choopun *et al*,2002). Serologically, more than 200 serogroups of *V.cholerae* have been identified, but only serogroup O1 and serogroup O139 have been reported to cause epidemic and pandemic cholera. The other non – O1,non-O139 serogroups are usually associated with some cases of mind gastroenteritis (Yael *et al*,2007).The sequencing of housekeeping genes may improve the current pragmatic definition of bacterial species (Stackebrandt *et al*,2002).Housekeeping genes *rec*A is a multifunctional protein contributing to homologous recombination, DNA repair and the sos response (Cox,2003;Lloyd&Sharp,1993). Thrompson*et al*(2004) analyzed the usefulness of *rec*A gene sequences as an alternative phylogenetic and/or identification marker to unravel phylogenetic relationships among higher taxonomic ranks because of its ubiquity and house-keeping function is bacterial (Zeigler,2003; Ludwig&Klenk, 2001;Eisen,1995). Therefore, to give insights about genetic relatedness between our isolates and the globally isolates reported elsewhere, the present study aimed at analyzing the *rec*A gene sequences for construction of phylogenetic trees analysis of *V.cholerae* in Iraq and in comparison to those recorded globally strains.

MATERIALS AND METHODS

Bacterial isolates

Twenty pathogenic *Vibrio cholera*e isolates were provided from Microbiology laboratory, which were isolated from patients suffering by diarrhea. These isolates were previously isolated by selective culture method and identified by Vitak biochemical reaction system.

Bacterial cultivation

Vibrio cholerae isolates were cultivated by inoculation on Brain Heart Infusion Broth media at 37°C overnight, then used in bacterial genomic DNA extraction.

Bacterial DNA extraction

The bacterial isolates were subjected to bacterial nucleic acid extraction by using commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA)**.** The extraction method was done depending on the manufacturing instructions by using gram positive bacteria DNA Protocol extraction method by using (10 mg/ml) proteinase K buffer.

Nanodrop

The extracted DNA was estimated by nanodrop device at 260/280nm, and then kept at deep freezer until used in PCR method.

Polymerase Chain Reaction

PCR technique was performed for detecting*rec*A gene in *Vibrio cholera*e isolates using specific primers designed for this study and this method was carried out according to Nei and Kumer (2000).

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Primers

The PCR primers that used in this study for detection *rec*A gene were designed in this study using NCBI Gene sequence data base gen bank code (EF990328.1) and primer 3 plus design. These primers were provided from Bioneer Company, Korea as following table (1):

Table (1): PCR primers and their sequence and GenBank codes

Primer	Sequence (5'-5')		Amplicon
*rec*A gene	F	TCQACCGGTTCTCTGTCTCT	516bp
	R	ACCGCCAGTGGTAGTTTCTG	

PCR master mix preparation

The mix was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depending on company instructions as following table (2)

Table (2): company instructions of PCR master mix

Master mix	Volume
DNA template (10 ng/µL)	5 µL
Forward Primer (10pmol)	1 µL
Reverse Primer (10pmol)	1 µL
PCR water	12 µL
Total Volume	20

After that, the PCR mix in table above placed in AccuPower PCR -PreMix that contains all other PCR components needed for reaction such as (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes were transferred into vortex centrifuge for 3 minutes and then transferred into thermocycler (MyGene, Bioneer. Korea).

PCR thermocycler conditions:

Table (3) :PCR thermocycler conditions

PCR step	Temp.	Time	Repeat
Initial Denaturatioin	95C	5min	1
Denaturatioin	95C	30sec	30 cycle
Annealing	60C	30sec	
Extension	72 C	1min	
Final extension	72C	5min	1
Hold	4C	Forever	-

PCR product analysis

The PCR products were examined by electrophoresis in a 1% agarose gel using 1X TBE buffer, stained with ethidium bromide, and investigation under UV transilluminator.

DNA sequencing method

The *rec*A gene PCR product was purified from agarose gel by using EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada. The purified PCR product samples were sent to Macrogen Company in Korea to performed DNA sequencing using *rec*A forward primer by AB DNA sequencing system. DNA sequencing method was performed for confirmative Phylogenetic tree relationship analysis of *Vibrio cholerae* based on *rec*A gene by Phylogenetic tree analysis using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

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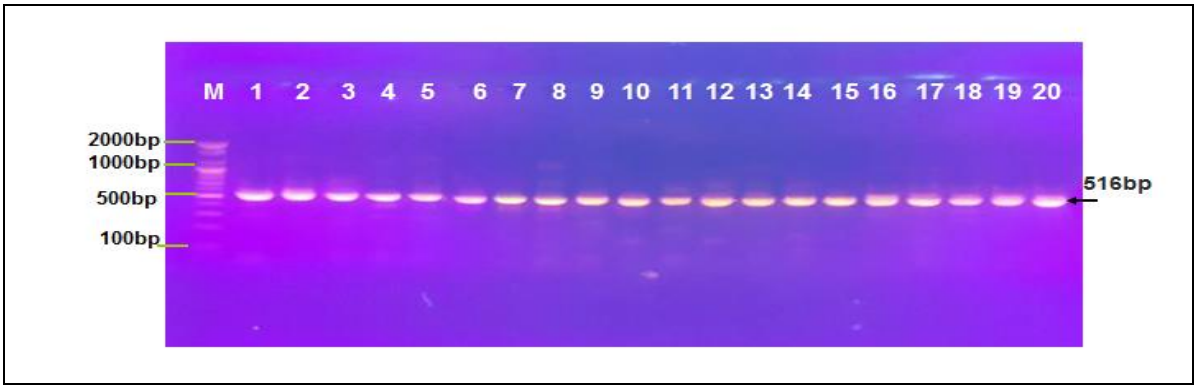
RESULTS AND DISCUSSION

The present study describe a molecular method for detection *Vibrio cholerae* isolates from human stool by using specific primer of *rec*A gene were designed in this study. Figure (1) shows presence of *rec*A gene in all *Vibrio cholerae* isolates. PCR assay was sensitive (100%) in comparison with (49%) sensitivity of direct bacterial culture (Zhang *et al*,2013). To date, *rec*A gene sequences have been used only to analyze *V.cholerae* strain (Stine *et al*,2000;Byun *et al*,1999).The *rec*A gene partial sequence for 20 local *Vibrio cholerae* IQ-D can be found under the accession numbers at NCBI-Gen Bank submission and they are shown in table (6).A562bp region of the *rec*A gene from 20 *Vibrio cholerae* isolates was obtained from human stool origins in Iraq, sequenced and compared with globally reported sequences. Results show alignment similarity and differences in *rec*A gene nucleotide sequences for 20 local *Vibrio cholerae* IQ-D isolates and five pathogenic NCB1-Blast Vibrio species by using (MEGA6)(Figure 2). In the present study, phylogenetic tree analysis was based on the *rec*A gene partial sequence that is used for confirmative detection analysis, the 20 local *vibrio cholerae* IQ-D isolates were closely related to NCBI-Blast *Vibrio cholerae* recA gene (U10162.1), whereas other NCBI-Blast pathogenic Vibrio species *rec*A gene were different out of tree at total genetic change (0.10 - 0.02%) (Figure 3). DNA sequencing technique benefits in comparing portions of genome from newly isolated bacterial with previous known bacterial strains which their sequence data available via online data bases (Belkum *et al*,2001). In this study, we estimated a pattern of nucleotide substitution in 20 local *Vibrio cholerae* isolates and five pathogenic NCB1-Blast *Vibriospecies* isolates by using (MEGA 6) as shown in table(4). Each entry in the table shows the probability of substitution (r) from one base (row) to another base (column)(Tajima, 1989).For simplicity, the sum of r values is made equal to 100.Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*, the nucleotide frequencies are 29.44% (A), 24.52% (T), 20.61% (C), and 25.44% (G).The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.All ambiguous positions were removed for each sequence pair. There were a total of 1411 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*,2013).The transition/transversion ratio test is important for determining the bias of substitution types and mutational patterns in a genome (Wakeley, 1996). These results agree with Teh*et al*(2011) who observed mutations in housekeeping and virulence genes in all serogroups of *Vibrio cholerae* strains with low amino acid substitution rates.



Figure(1): Agarose gel electrophoresis image that shown the PCR product of recA gene in *Vibrio cholerae* isolates. Where M: Marker (2000-100bp), lane (1-20) positive amplification at (516bp) PCR product.

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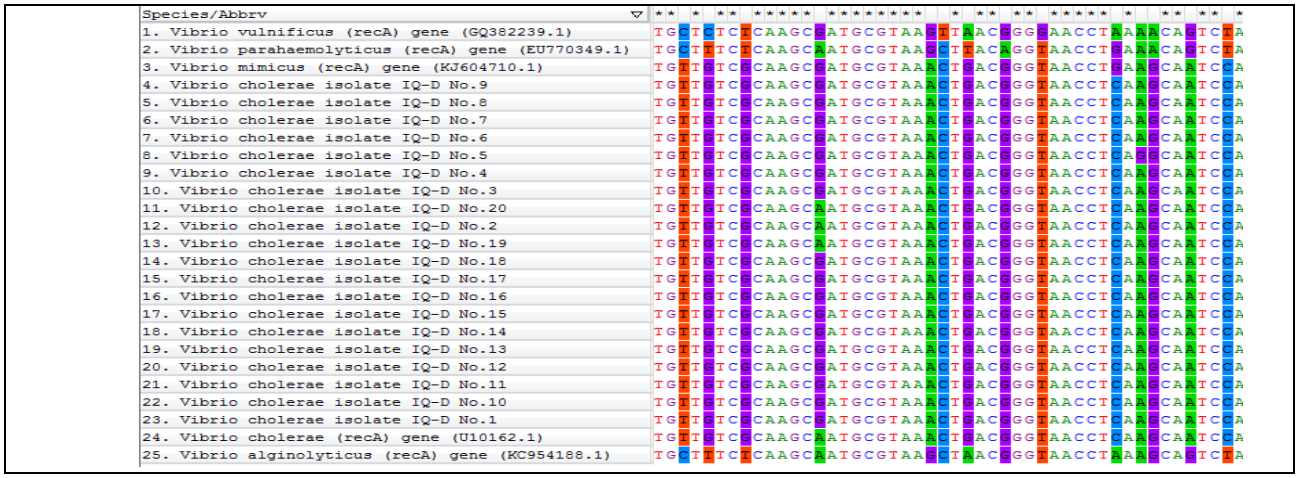

Figure (2): Multiple sequence alignment analysis of *rec*A gene partial sequence for local *Vibrio cholerae* IQ-D (20 isolates and five pathogenic NCBI-Blast Vibrio species isolates by using (MEGA 6.0, multiple alignment analysis tool).

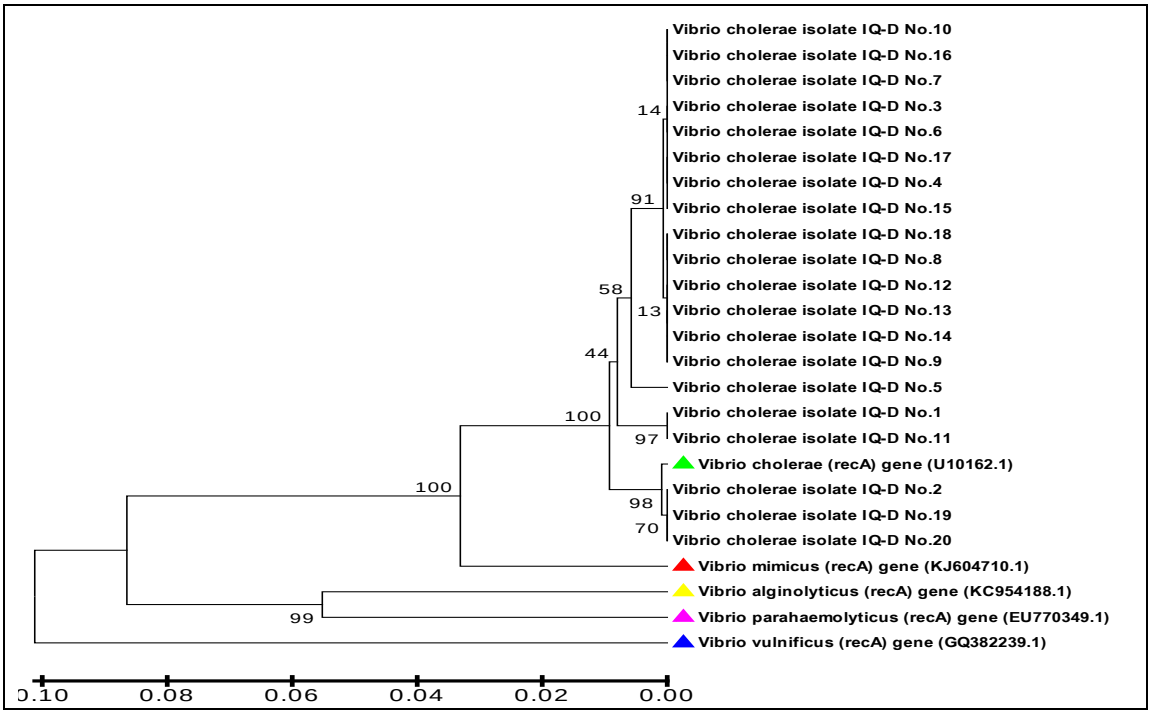

Figure (3): Phylogenetic tree analysis based on the *rec*A gene partial sequence that used for confirmative detection analysis. The phylogenetic tree was constructed using (UPGMA tree) in (MEGA 6.0 version).

Table (4): Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*1.56*	*1.31*	**25.14**
T	*1.88*	-	**15.08**	*1.62*
C	*1.88*	**17.94**	-	*1.62*
G	**29.09**	*1.56*	*1.31*	-

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Results show alignment similarity and differences in *rec*A gene nucleotide sequences for 20 local vibrio cholera IQ-D isolates and other NCBI-Blast vibrio cholera isolates by using (MEGA6) (Figure 4). The high similarity of genomes found among different species of vibriosis may be explained by niche adaptation (Cohan,2004). In addition, although *rec*A are thought to belong to bacterial core genome and for this reason may be refractory to horizontal gene transfere(HGT)(Harris *et al*,2003).In the present study, phylogenetic tree analysis is based on the *rec*A gene partial sequence that is used for genetic diversity analysis. The 20 local vibrio cholera IQ-D isolates show more difference than NCBI-Blast vibrio cholera isolates at total genetic change (8-2%) (Figure5). The housekeeping genes are good candidates for the determination of genetic relatedness because of their diversity slowly and appear only limited variation in their sequence (Danine-Pelego*et al*,2007). Therefore, *rec*A sequencing is available analysis method for understanding the relatedness of the local isolates with the isolates obtained elsewhere (Dashtbani-Roozbehani *et al*,2011). Pattern of nucleotide substitution was eliminated in 20 local Vibrio isolates and seven pathogenic NCBI-Blast *Vibrio cholera* were isolates by using MEGA6. Rates of different transitional substitution are (17.11,13.85,16.3,19.16) and rates of transversional substitution are (4.12 , 3.5 ,4.84, 4.32 , 4.84 ,4.32, 4.12 , 3.5) (Table5).*Rec*A gene is highly vulnerable to the ecological pressure resulting in dispersed point mutations throughout the *rec*A, yet unchanging the structure and overall functionality of *rec*A (Dashtbani-Roozbehani*et al*,2011). Genetic diversity of 20 local *vibrio cholerae* isolates and the highest genetic diversity value show in *vibrio cholerae* isolate IQ-D No.3 (ky766037). The genetic diversity rate of *rec*A gene could serve as individual predictor gene for genome relatedness because of it reflects the total genome difference (Zeigler *et al*,2011).

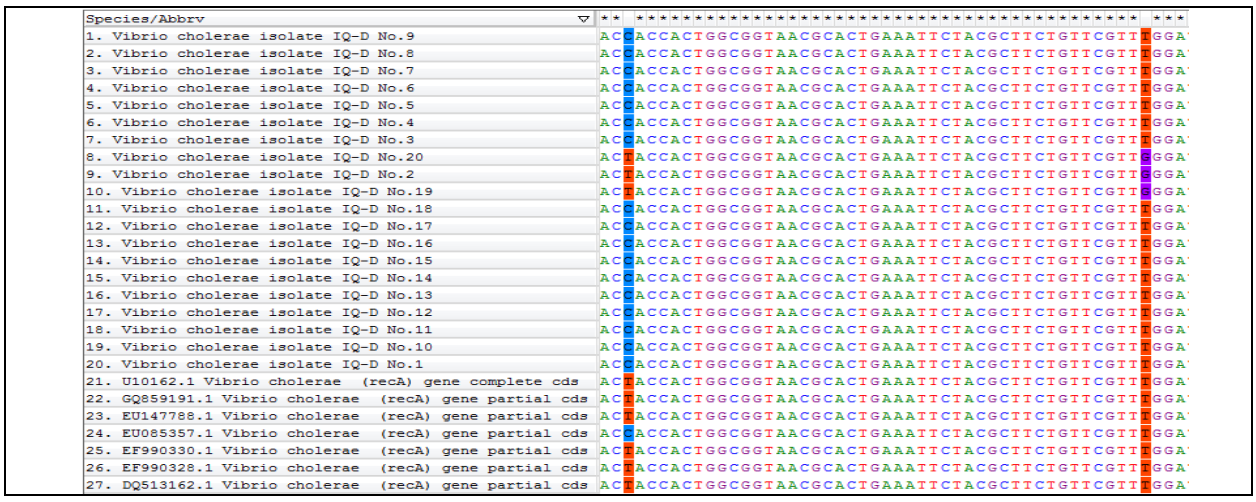

Figure (4): Multiple sequence alignment analysis of *rec*A gene partial sequence for local *Vibrio cholerae* IQ-D (20 isolates and other NCBI-Blast *Vibrio cholerae* isolates by using MEGA 6.0 multiple alignment analysis tool) shows the alignment similarities (*) and differences in *rec*A gene nucleotide sequences.

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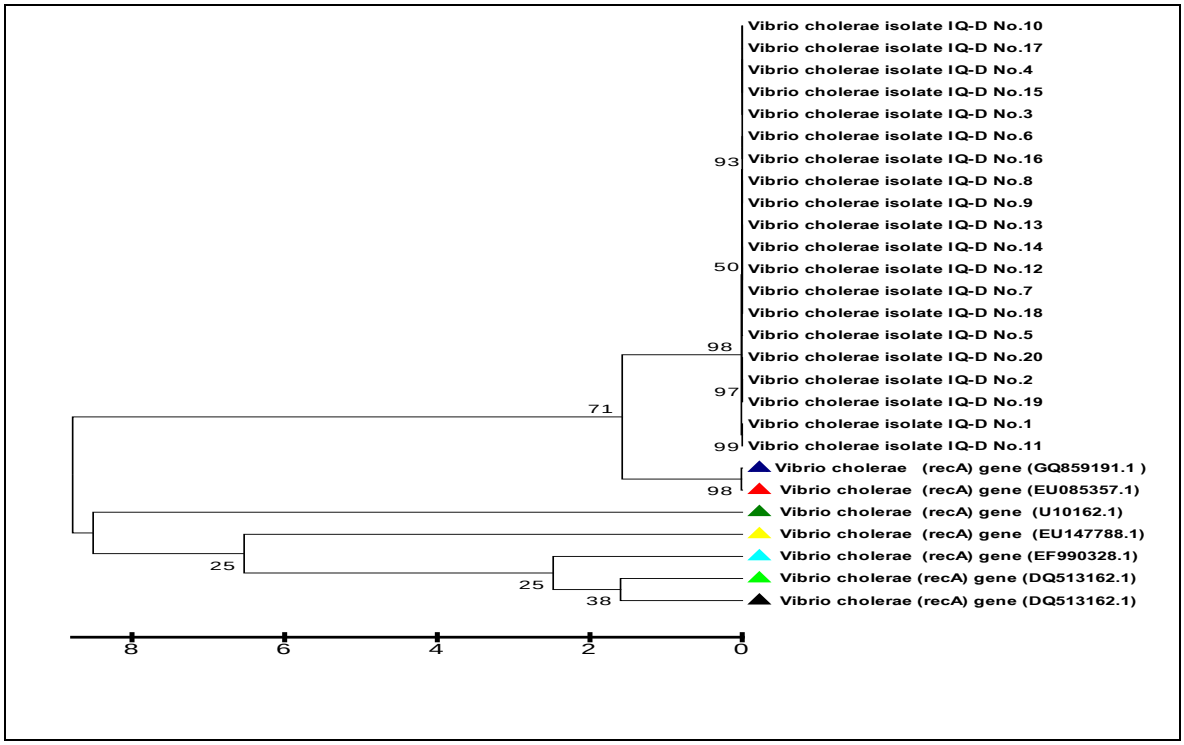

Figure (5: Phylogenetic tree analysis based on the *rec*A gene partial sequence that used for genetic diversity analysis. The phylogenetic tree was constructed using (UPGMA tree) in (MEGA 6.0 version).

Table (5): Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*4.12*	*3.5*	**17.11**
T	*4.84*	-	**13.85**	*4.32*
C	*4.84*	**16.3**	-	*4.32*
G	**19.16**	*4.12*	*3.5*	-

Table (6): Genetic diversity of local *Vibrio cholerae* isolates and NCBI-Blast *Vibrio cholerae* isolates.

Local isolates No.	Genbank accession number	Genetic diversity index (π)
Vibrio cholerae isolate IQ-D No.1	KY766035	0.003980
Vibrio cholerae isolate IQ-D No.2	KY766036	0.008473
Vibrio cholerae isolate IQ-D No.3	KY766037	0.048142
Vibrio cholerae isolate IQ-D No.4	KY766038	0.004303
Vibrio cholerae isolate IQ-D No.5	KY766039	0.006606
Vibrio cholerae isolate IQ-D No.6	KY766040	0.004303
Vibrio cholerae isolate IQ-D No.7	KY766041	0.006353
Vibrio cholerae isolate IQ-D No.8	KY766042	0.006353
Vibrio cholerae isolate IQ-D No.9	KY766043	0.004455
Vibrio cholerae isolate IQ-D No.10	KY766044	0.004303
Vibrio cholerae isolate IQ-D No.11	KY766045	0.006733
Vibrio cholerae isolate IQ-D No.12	KY766046	0.004455

***Vibrio cholerae* isolate IQ-D No.13**	**KY766047**	**0.004455**
***Vibrio cholerae* isolate IQ-D No.14**	**KY766048**	**0.004455**
***Vibrio cholerae* isolate IQ-D No.15**	**KY766049**	**0.004303**
***Vibrio cholerae* isolate IQ-D No.16**	**KY766050**	**0.004303**
***Vibrio cholerae* isolate IQ-D No.17**	**KY766051**	**0.004303**
***Vibrio cholerae* isolate IQ-D No.18**	**KY766052**	**0.004455**
***Vibrio cholerae* isolate IQ-D No.19**	**KY766053**	**0.003923**
***Vibrio cholerae* isolate IQ-D No.20**	**KY766054**	**0.003923**

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