Molecular detection and phylogenetic analysis of *Vibrio cholerae* genotypes in Hillah, Iraq

A. T. Al-Sa'ady[1], K. A. Baqer[2] and Z. K. S. Al-Salim[2]

1) *Department of Clinical Laboratory Sciences, Faculty of Pharmacy, University of Babylon* and 2) *Department of Microbiology, Al Imam Al Sadiq Teaching Hospital, Hillah, Iraq*

Abstract

Vibrio cholerae is a cause of serious endemic diarrhoea associated with cholera in many regions in the world. A total of 256 stool and rectal swabs were collected from patients suspected to have cholera admitted to three hospitals in Hillah, Babylon Governorate, Iraq, for the period 1 September to 29 December 2017. After the routine culture of samples for isolation and identification of *V. cholerae* isolates, PCR was performed for molecular detection of *V. cholerae* isolates based on 16S ribosomal RNA gene. Toxigenicity was detected by RTX toxin genes. PCR technique emphasized molecular detection of *V. cholerae* for eight isolates. Only two isolates (25%) possessed both the *rtxA* and *rtxC* genes, while only three isolates (37.5%) possessed the *rtxB* gene. DNA sequencing was performed for the eight isolates via analysis and phylogenetic tree. The observed bacterial variants were compared to their neighbour homologous reference sequences using the National Center for Biotechnology Information (NCBI) BLAST server (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The findings indicated that the eight investigated isolates of *V. cholerae* were positioned in three different phylogenetic positions. Partial sequence dissimilarities were reported between GenBank isolate accession number MK212155.1 and these six clustered GenBank accession numbers of the same species. For the first time in Babylon Governorate, Iraq, the molecular assay, sequencing and phylogenetic tree are reported for *V. cholerae* and their toxins isolated during the 2017 cholera outbreak.
© 2020 The Authors. Published by Elsevier Ltd.

Corresponding author. A. T. Al-Sa'ady.
E-mail: amal.atiyah@yahoo.com

Introduction

Recently, in 2007, 2009 and 2012, periodic outbreaks of cholera were recorded, with outbreaks starting in July with an increase in acute diarrhoea (AD) that reached its peak in September. The most recent cholera outbreaks were officially declared in Iraq in 2015 and 2017 [1−4]. A report by the EWARN system in Iraq in May 2017 documented the trends of AD reported in Iraq in week 1 to week 21, and indicated that the proportion of AD cases slightly increased since the last weeks (week 20, 7.9%; week 21, 8%) (Fig. 1) [5]. In Ninewa Governorate, Iraq, at 4%, AD (18/435) represented the second most common disease reported from 1 to 30 April 2017 [6]. This percentage increased to 10% (27/894) from 1 to 31 May 2017 [7]. From 1 to 30 November 2017, 162 suspected cholera cases were reported, including 161 suspected cases in Ninewa, five of which were verified to be positive, and one suspected case in Dahuk, which was verified to be falsely positive [8]. AD was still the second most common disease reported in Iraq overall in December, at 3% (17 692 cases) (Fig. 2) [9]. Hussein [4] tried to determine the infection rate of *Vibrio cholerae* in Iraq during 2017 and reported that the majority, at 63.75%, to be in Baghdad−Al Risafa, 19.88% in Babylon, 14.04% in Wassit, 1.75% in Diwaniyah and 0.58% in Najaf. In 2018, some cases of cholera were also reported in Iraq [10].

A lack of clean drinking water in Iraq represents a major health problem, as cholera is transmitted by contaminated water. Cholera's incubation period ranges from hours to a few days; the most

common symptom is severe watery diarrhoea. Once *V. cholerae* enters the intestines, the secretion of cholera toxins will start, which leads to the characteristic diarrhoea as well as vomiting, resulting in severe dehydration from loss of water and electrolytes; dehydration can reach up to 15 L a day. This diarrhoeal stool which was contaminated by *V. cholerae* will release in large quantities into the environment, resulting in the spread of this bacterium and its transmission via the faecal−oral route. Within 1 to 3 days of infection, without suitable treatment, 25% to 50% of patients may die as a result of circulatory collapse or a steep drop in blood pressure. The highest death rate has been recorded among children, the elderly and immunocompromised people [11−13].

Vibrio cholerae is a cause of serious pandemic diarrhoea associated with cholera in many regions in the world; managing illnesses due to this microorganism is a remarkable problem associated with the life-threatening cholera toxins. Recently a novel cholera toxin was found to be a member of the repeats in toxin (RTX) family of toxins, which represent essential virulence factors. RTX toxins comprise a group of bacterial cytolysins and cytotoxins. The abbreviation RTX refers to 'repeats in toxin' because of the repeating of the glycine- and aspartate-rich areas located at the C terminus of the toxin proteins, which facilitate export by a dedicated type 1 secretion system encoded within the *rtx* operon [14−16].

Depending on the specificity of the target cell, RTX toxins are subclassified into two groups, haemolysins and leukotoxins. A third group of RTX toxins with multiple activities was discovered in 1999. RTX toxin is encoded by the general *rtx* gene cluster. In *V. cholerae*, the cytotoxin (*rtxA*), the acyltransferase (*rtxC*) and an associated ATP-binding cassette transporter system (*rtxB*) are encoded by the RTX toxin gene cluster [17−19].
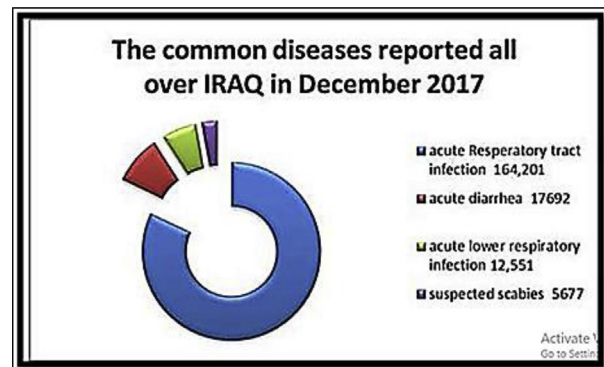

FIG. 2. Common diseases reported all over Iraq in December 2017 [9].

The genetic material of bacteria is predisposed to mutability due to mutation or recombination. The sequence variability within particular genes can be used in molecular analyses to determine the relatedness of bacteria. Roetzer et al. [20] suggested that whole genome sequencing was better than conventional genotyping for pathogen tracing and investigating microepidemics. Whole genome sequencing provides a measure of genome evolution over time in its natural host context. Tamura et al. [21] explained that comparative analysis of molecular sequence data was essential for reconstructing the evolutionary histories of species and for inferring the nature and extent of selective forces shaping the evolution of genes and species as well as analyses for inferring evolutionary trees, selecting best-fit substitution models (nucleotide or amino acid), inferring ancestral states, identifying sequences (along with probabilities) and estimating evolutionary rates site by site.
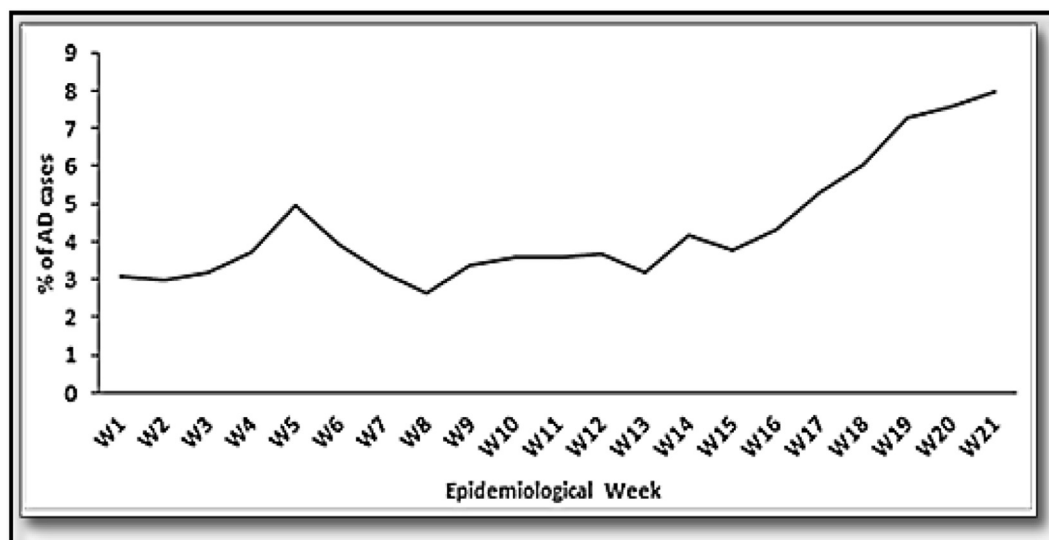

FIG. 1. Proportion of cases of acute diarrhoea (AD) reported by week for weeks 1 to 21, 2017, Iraq [5].

Babylon Governorate, like the rest of the Iraqi governorates, has experienced several cholera outbreaks in the last few years. We found no Iraqi studies that dealt with the genetic and molecular diagnoses of *V. cholerae*, their toxins and their genetic sequences. We therefore aimed to gain more in-depth knowledge regarding the molecular characterization, sequencing and phylogenetic tree of *V. cholerae* in Babylon, Iraq.

Materials and methods

Sample collection

A total of 256 samples (196 stool samples and 60 rectal swabs) were collected from patients admitted to the outpatient clinics in Al-Hillah General Teaching Hospital, Babylon Hospital for Pediatric and Gynecology and Al Imam Al Sadiq Teaching Hospital in Hillah, Babylon Governorate, Iraq, who were treated from 1 September to 29 December 2017. The samples were collected by a physician before treatment was provided to patients. The samples came from people aged 20 to 60 years who were suspected of having cholera by a specialist clinical physician. Samples were transported in Cary-Blair transport medium to the laboratory and inoculated in alkaline peptone water for 4 to 6 hours at 35°C, then cultured on blood agar, MacConkey agar and selective thiosulfate citrate bile salt sucrose agar at 37°C for 24 hours. The isolation and presumptive diagnosis of *V. cholerae* isolates relied on the protocol provided by the Central Public Health Laboratory (CPHL) of the Iraqi Ministry of Health. The VITEK 2 system (bioMérieux, Marcy l'Étoile, France) was used to confirm the diagnosis.

We gained permission from the authority of Al-Hillah General Teaching Hospital, Babylon Hospital for Pediatric and Gynecology and Al Imam Al Sadiq Teaching Hospital in Babylon Governorate, Iraq, to collect the samples, together with verbal consent from patients and/or their relatives to take samples from them for scientific purpose while maintaining the safety of the patients and respecting their privacy.

Molecular assay

DNA purification of *V. cholerae* isolates were performed according to the genomic DNA purification protocol supplied by the manufacturing company (Presto Mini-DNA Bacteria Kit; Geneaid Biotech, New Taipei City, Taiwan). The NanoDrop UV spectrophotometer (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) was used to estimate the extracted DNA at 260 and 280 nm and kept it frozen. PCR was performed for molecular detection of *V. cholerae* isolates based on 16S ribosomal RNA (rRNA) gene and identification their RTX toxin genes according to method described by Chow et al. [22]. In the present study, the PCR primers for the 16S rRNA gene were designed for this study according to database AY513501.1 in the NCBI's Gene sequence.

The other primers were provided by Macrogen (Seoul, Korea) for RTX toxin genes according to Chow et al. (Table 1).

The T100 Thermal thermocycler (Bio-Rad, Hercules, CA, USA) was used for PCR. The conditions of PCR included one cycle at 95°C for 5 minutes, then 30 cycles at 95°C for 30 seconds; the annealing included 30 cycles for 30 seconds at 58°C for the 16S rRNA gene and at 55°C for RTX toxin genes. Then conditions included 30 cycles at 72°C for 1 minute and one cycle at 72°C for 5 minutes; the final product was kept at 4°C. Agarose gel electrophoresis was used to confirm the successful PCR amplification. A UV transilluminator was used for the observation of DNA bands.

DNA sequence and phylogenetic analysis

The 16S rRNA PCR product of *V. cholerae* isolates was purified from agarose gel (EZ-10 Spin Column DNA Gel Extraction Kit; Biobasic, Markham, Canada). The purified 16S rRNA gene PCR product samples were sent to Macrogen in Korea to perform the DNA sequencing using an 16S rRNA forward primer by the AB DNA sequencing system (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA). A specific comprehensive tree was constructed according to the neighbour-joining protocol described by Sarhan et al. [23], with some modifications. The observed variants were compared with the deposited reference sequences using the NCBI-BLAST server. Next, the BLAST results of the observed variants were combined and aligned by the neighbour-joining method with gap corrections using Clustal Omega−based tools [24]. Then, a full inclusive tree, including the observed variant, was annotated and visualized as an unrooted tree using iTOL software [25]. The sequences of each classified phylogenetic species group in the comprehensive tree were coloured appropriately.

Results

Isolation and identification

Depending on the protocol provided by the CPHL, *V. cholerae* isolates were isolated and diagnosed presumptively (Fig. 3).

TABLE 1. PCR primer sequences and product size

Oligonucleotide primer	Nucleotide sequence (5′−3′)	Product size (bp)
16S rRNA gene	TCTGAGACAGGTGCTGCATG GCTTCTTTTGCAGCCCACTC	400
rtxA gene	CTGAATATGAGTGGGTGACTTACG GTGTATTGTTCGATATCCGCTACG	417
rtxC gene	CGACGAAGATCATTGACGAC CATCGTCGTTATGTGGTTGC	263
ctxB gene	GATACACATAATAGAATTAAGGATG TCGAACCACGGTTGCTTCTCATCA	460

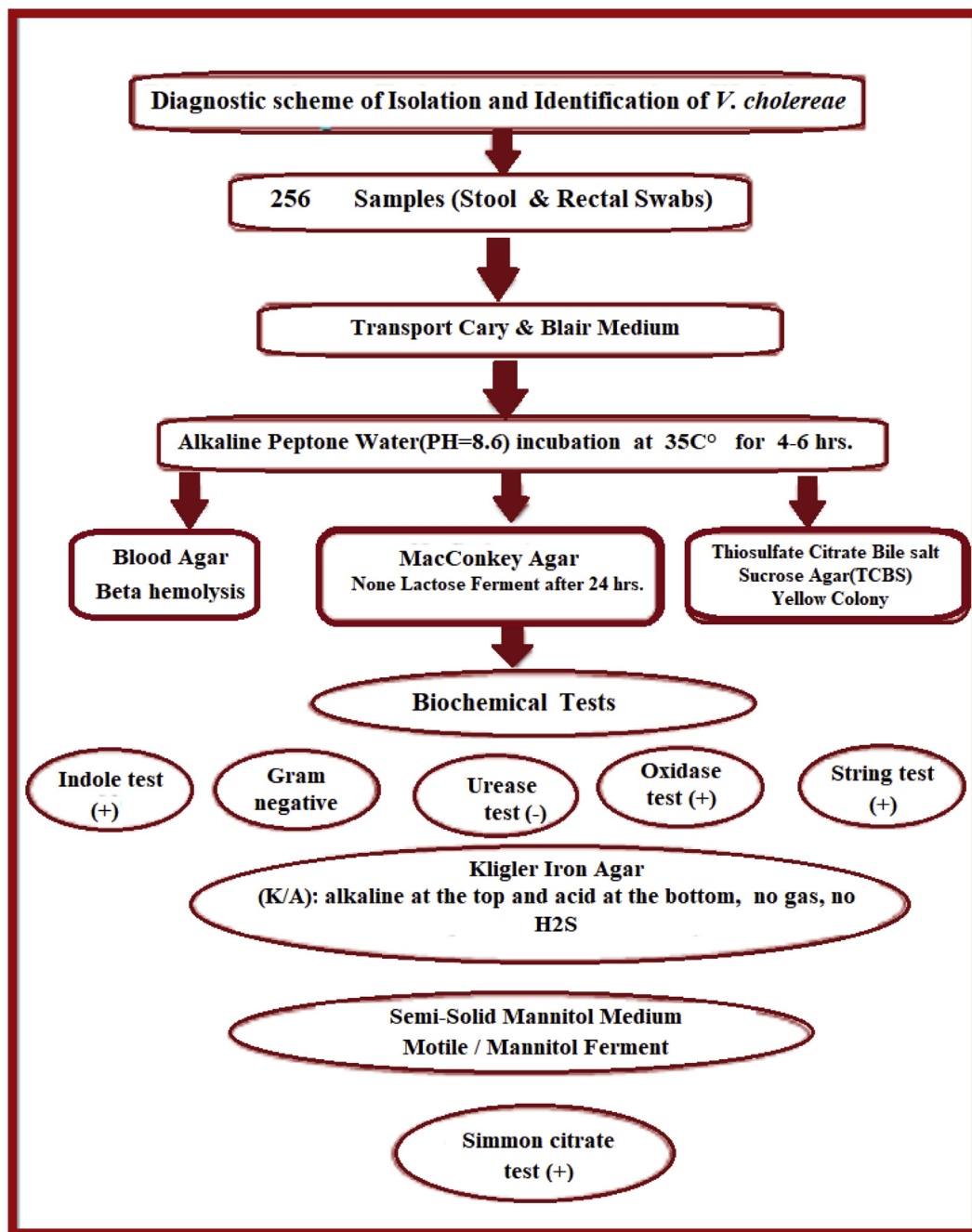Purchased from Macrogen, Seoul, Korea.

FIG. 3. Diagnosis scheme of isolation and identification of *Vibrio cholerae* in present study depending on protocol provided by Central Public Health Laboratory (CPHL), Iraqi Ministry of Health.

Nineteen isolates had positive results for *V. cholerae*, but after confirmation of the identification by the VITEK 2 system, only nine isolates of *V. cholerae* were obtained. The rest of the isolates were Gram-negative bacteria and are not included in the current study.

Molecular assay

Our molecular analysis by PCR emphasized the molecular detection of *V. cholerae* on the basis of the presence of the 16S

rRNA gene; detection of three RTX toxin genes included the *rtxA* gene, *rtxB* gene and *rtxC* gene. As detailed in Table 2, among a total number of nine isolates identified by the VITEK 2 system, only eight (isolates 1, 2, 3, 4, 5, 7, 8 and 9) had positive test results for *V. cholerae* by PCR with a product size of 400 bp, whereas isolate 6 had a negative result (Fig. 4), thereby further emphasizing that isolate 6 was not *V. cholerae*. It was therefore excluded from the next molecular experiments in this study. As

TABLE 2. Molecular detection of *Vibrio cholerae*

Isolate no.	16S rRNA gene	*rtxA* gene	*rtxC* gene	*rtxB* gene
1	+	—	—	—
2	+	—	—	—
3	+	—	—	—
4	+	—	—	—
5	+	—	—	—
6	—	Excluded	Excluded	Excluded
7	+	+	+	+
8	+	—	—	+
9	+	+	+	+

rRNA, ribosomal RNA; *rtx*, repeats in toxin.

detailed in Table 2, among eight isolates of *V. cholerae*, only two (25%), isolates 7 and 9, had a positive result for each *rtxA* gene (417 bp) and *rtxC* gene (263 bp) (Figs. 5 and 6). Only three isolates (37.5%), isolates 7, 8 and 9, had positive PCR test results for the *rtxB* gene (460 bp) (Fig. 7).

Sequence of *V. cholerae* 16S rRNA gene

The results of gene sequencing for the 16S rRNA gene revealed that 100% identification of isolate 5 when compared its sequence to those of the global isolates in the NCBI-BLAST database, with 0% gaps, while detected 99% identification for isolates numbered 1, 2, 3, 4, 5, 7, 8 and 9 (Table 3).

DNA sequencing analysis based on a partial sequence of the 16S rRNA gene was found to have a phylogenetic relationship and homology sequence identity between local *V. cholerae* isolates and NCBI-BLAST isolates. As shown in Fig. 8, multiple sequence alignment analysis of the 16S rRNA gene and partial sequence of local *V. cholerae* isolates showed a similarity in genetic arraying between isolates around the world as a result of conservation of DNA over the years, with slight differences and changes due to horizontal gene transmission.

Fig. 9 shows the phylogenetic tree we constructed using the unweighted pair group method with arithmetic mean (UPGMA tree) in MEGA 6.0 software. The local *Vibrio cholerae* (samples 1, 4, 7 and 9) were found to be related to NCBI-BLAST *V. cholerae* isolates, and local *V. cholerae* sample 5 was found to be closely related to NCBI-BLAST *V. cholerae* isolate KY084548.1. Local *V. cholerae* isolate 8 showed some genetic differences and was out of tree, with a total genetic change of 0.0005% to 0.003%.

Phylogenetic tree of the 16S rRNA gene of *Vibrio cholerae*

As detailed in Fig. 10, a comprehensive tree of genetic variants of the 16S rRNA fragment for eight *Vibrio cholerae* local isolates was constructed. The total number of the aligned nucleic acid sequences, irrespective of the investigated bacterial variants, in this comprehensive tree was 84 sequences. Thus, the total number of sequences within the currently constructed
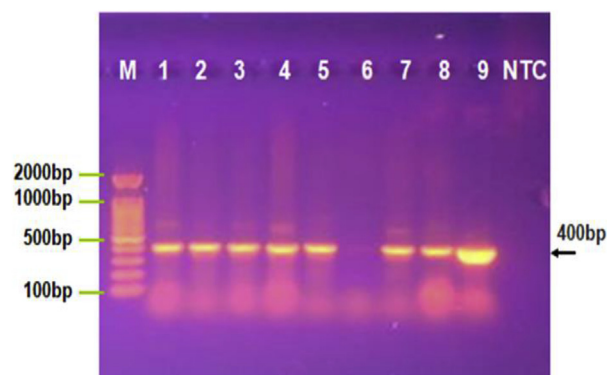

FIG. 4. Agarose gel electrophoresis for PCR product of 16S ribosomal RNA gene for confirmatory molecular detection of *Vibrio cholerae* at product size 400 bp. Lanes 1, 2, 3, 4, 5, 7, 8 and 9 are positive for *V. cholerae* isolates; lane 6 is negative. M, marker (100−1500 bp); lane, nontemplate negative control (NTC).

phylogenetic tree was 92 sequences, with the presently investigated isolates aligned side by side with other related sequences within the tree in an unrooted pattern.

Discussion

Vibrio cholerae is the aetiologic agent of cholera. It is Gram-negative, curved, motile, noninvasive rod and a member of the *Vibrionaceae* family. *V. cholerae* has a multifactorial pathogenesis, including virulence factors and toxic action. After the microorganism colonizes the human intestine, cholera toxins are produced, resulting in the disease's diarrhoeal syndrome. In addition, other toxins, such as RTX, play a significant role in the pathogenesis with haemolytic, leukotoxic and leukocyte-stimulating activities. In *V. cholerae*, the RTX gene cluster

FIG. 5. Agarose gel electrophoresis PCR product of *rtxA* gene in *Vibrio cholerae* at product size 417 bp. Only lanes 7 and 9 are positive; other lanes are negative. M, marker (100−1500 bp).

FIG. 6. Agarose gel electrophoresis for PCR product of *rtxC* gene in *Vibrio cholerae* (263 bp). Only lanes 7 and 9 are positive; other lanes are negative. M, marker (100−1500 bp).

plays a major role in cytotoxic activity and involves four genes (*rtxA−D*) located on the large chromosome near the *ctx* genes. Depending on the somatic O surface antigen, *Vibrio cholerae* may be classified into four serogroups; O1 and O139 serogroups are associated with cholera, while non-O1 and non-O139 serogroups are associated with cholera-like diarrhoea [11,12,26,27].

Molecular assay

As detailed in Table 2, the positive PCR result for the 16S rRNA gene was reported in eight (89%) of nine isolates for the molecular diagnosis of *V. cholerae* (Fig. 4). This finding is in agreement with many studies which used the 16S rRNA gene for identification of *Vibrio* spp. The molecular diagnosis of pathogenic bacteria to the species level is essential to determine the exact aetiologic agent of any outbreak. PCR helps researchers avoid the potential difficulties associated with using conventional identification techniques to discriminate among
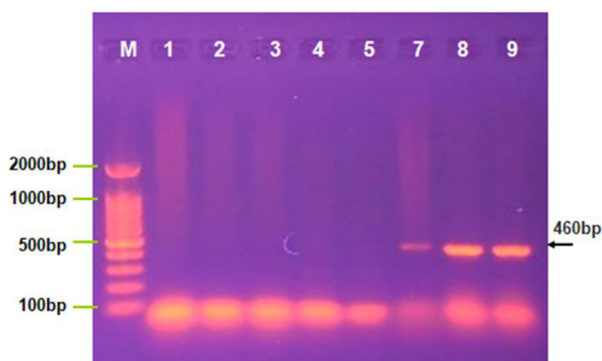
V. cholerae isolates. Although, the isolates possess rtx genes, perhaps could not be found by routine tests to express cholera toxins. SO, the 16S rRNA gene has been needed for the species-specific identification of Vibrio spp. In addition, the best detection for V. cholerae toxigenicity may be done by PCR technique and primers during specific amplification for genes encoded for cholera toxins [28−31].

The 16S rRNA gene sequences were used to study the phylogenetic and taxonomic structures of the bacterium; indeed, they comprise the most common bacterial molecular marker because the 16S rRNA gene is present in nearly all bacterial species and has a function that has remained unchanged. Further, the large sequence of this gene makes it suitable for diagnostic purposes. Many bacterial genera and species are hard to identify because they do not fit any known biochemical or commercial parameters, whereas studying the 16S rRNA gene permits us to obtain an exact identification [32−36].

As detailed in Table 2, in the present study, only two isolates (isolates 7 and 9) had both the *rtxA* gene (Fig. 5) and the *rtxC* gene (Fig. 6). Only three isolates (isolates 7, 8 and 9) had the *rtxB* gene (Fig. 7). This finding is in agreement with Siriphap et al. [37], who found that 100% of clinical and environmental *V. cholerae* isolates in Thailand had the *rtxA* gene. The *rtxA* toxin in *V. cholerae* is a member of the RTX family of pore-forming toxins and has the ability to cause depolymerization of actin stress fibers, resulting in rounding of cells in culture without any cytolytic or haemolytic activity [17].

A study in Hong Kong of isolates gathered from 1986 to 1999 reported that all 166 isolates of *V. cholerae* had both the *rtxA* gene and the *rtxC* gene. Both genes were accompanied by the ability of *V. cholerae* to express phenotypic and cytotoxic activity in all the studied isolates. Negative amplification of PCR in some isolates may be ascribed to the deletion of the gene cluster [22]. Also, in a study by Goel et al. [36] in India, all isolates of *V. cholerae* had positive PCR results for the *rtxC* gene. A study conducted in Yunnan province, southwest China, by Liao et al. [38] found that the PCR and sequence results emphasized that all isolates of *V. cholerae* possessed the *rtxB* gene.

Previous studies have indicated the basic action of the three studied genes, *ctx*A, *ctx*B and *rtx*C, together with *V. cholerae* toxigenicity. A study by Boardman and Satchell [39] showed that the *V. cholerae* RTX toxins are secreted by a type 1 secretion system encoded by *rtx*B, *rtx*D, *rtx*E and *tolC*. The mutations in both *rtx*B and *rtx*E blocked the secretion of these toxins. *In vitro*, a gene cluster in El Tor *V. cholerae* was characterized consisting of four genes: *ctx*A, *ctx*B, *rtx*C and *rtx*D. All these genes are required for cytotoxic activity and encode for cholera RTX toxins. Unfortunately, some residual adverse

FIG. 7. Agarose gel electrophoresis for PCR product of *ctx*B gene in *Vibrio cholerae* (460 bp). Only lanes 7, 8 and 9 are positive; other lanes are negative. M, marker (1500−100 bp).

TABLE 3. NCBI-BLAST homology sequence identity (%) between local *Vibrio cholerae* 16S rRNA isolates and NCBI-BLAST submitted *Vibrio cholerae* 16S rRNA gene isolates

Local *V. cholerae* isolate no.	Isolate source	GenBank accession no.	NCBI-BLAST homology sequence identity (%) for *V. cholerae* sample:			
			KY084548.1	KY474109.1	KY474110.1	MH244240.1
1	Stool	MK212151	99%	99%	99%	99%
2	Stool	MK212152	99%	99%	99%	99%
3	Stool	MK212153	99%	99%	99%	99%
4	Stool	MK212154	99%	99%	99%	99%
5	Stool	MK212155	100%	100%	100%	100%
7	Stool	MK212156	99%	99%	99%	99%
8	Stool	MK212157	99%	99%	99%	99%
9	Stool	MK212158	99%	99%	99%	99%

NCBI-BLAST, National Center for Biotechnology Information Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi); rRNA, ribosomal RNA.

properties associated with the attenuated cholera vaccines may be related to RTX toxins [39−41].

A study by Chatterjee et al. [43] demonstrated that these RTX toxins are important factors in *V. cholerae* pathogenesis. Previous studies have confirmed that a high rate of *V. cholerae* isolated from the environment has the *rtx* gene cluster [44,45]. Xu et al. [46] found high percentages of the *rtxA* (83.0%), *rtxB* (97.0%), *rtxC* (95.8%) and *rtxD* (95.5%) genes from *V. cholerae* isolated from freshwater fish. Related studies have emphasized that *V. cholerae* can survive a long time in the environment, which is why Iraq's cholera outbreaks are associated with seasonal changes in climate and temperature as well as algae blooms [47−50]. In Iraq, many factors must be taken into consideration while studying the spread of cholera, including the source of the water supply, socioeconomic status, living conditions, high population density, poor hygiene, poor food safety, cultural beliefs and practices, levels of education, status of the immune system, age and gender. Interestingly, a brief description of the effects of these factors is provided in an Iraqi study by Karim and Darwish [51], who concluded that the outbreak in Iraq during 2017 was cholera associated with factors like being female, being 15 to 45 years old and living in a highly populated residential area with poor sanitation systems and a bad source of water (tap water, or reverse osmosis−treated water provided from local portable distributors).

Sequence and phylogenetic analysis of the 16S rRNA gene in *V. cholerae*

For complete detection of *V. cholerae*, the sequence result needs to be analysed and compared with those in GenBank to find out the sequence differences using NCBI-BLAST. The results of the DNA sequencing should be provided at the beginning of the test to confirm that the nucleotide sequences have a close relationship with other world strains [51,52]. Analysing the phylogenetic pattern is a good protocol for the detection of functional prediction and evolutionary trends. Phylogenetic trees have many functions; the generation of branching, treelike diagrams provides a way to visualize an estimated lineage of the genetic relationship among organisms,
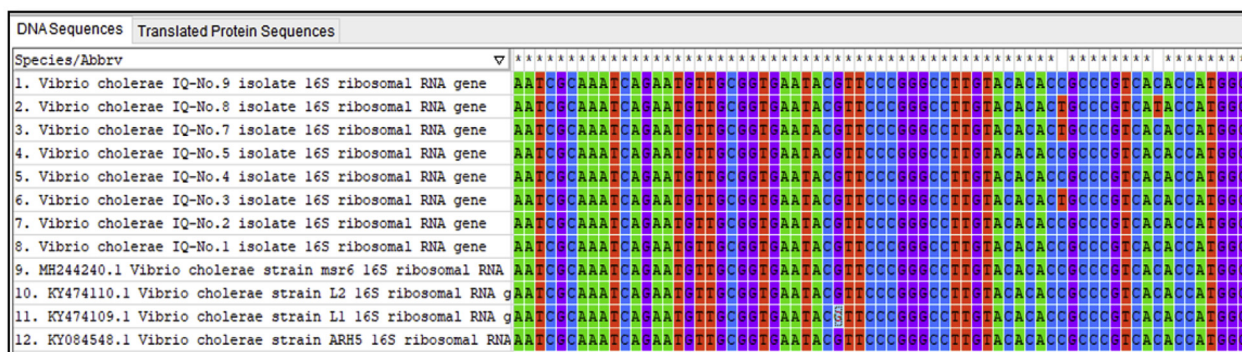

FIG. 8. Multiple sequence alignment analysis of 16S ribosomal RNA gene partial sequence for local *Vibrio cholerae* (isolates 1−5 and 7−9) with NCBI-BLAST of *V. cholerae* isolates 16S ribosomal RNA gene (NCBI-BLAST; National Center for Biotechnology Information Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment analysis was constructed by Clustal W alignment tool in MEGA 6.0. Results show nucleotide alignment similarity (*) with different *V. cholerae* isolates.
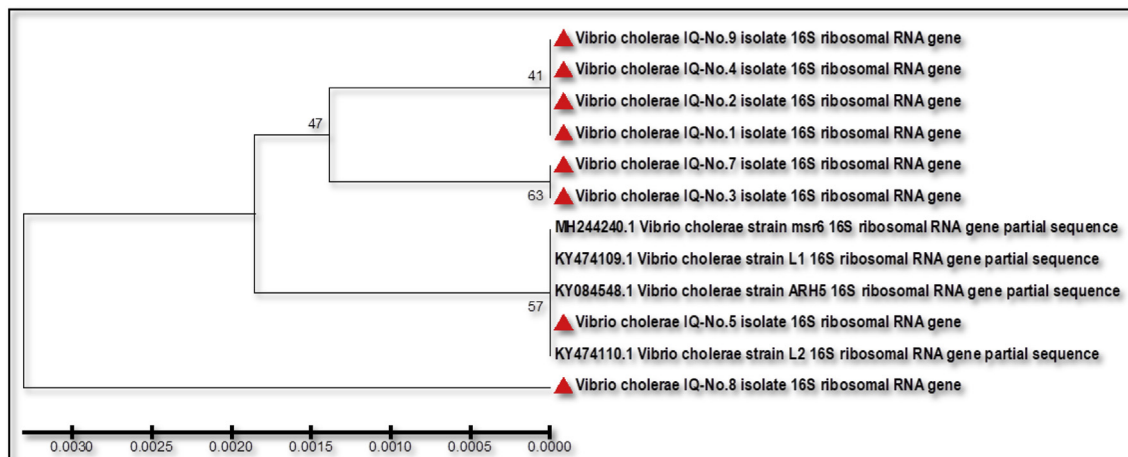
FIG. 9. Phylogenetic tree analysis based on 16S ribosomal RNA gene partial sequence used for confirmatory analysis of local *Vibrio cholerae* using unweighted pair group method with arithmetic mean (UPGMA tree) in MEGA 6.0.

showing the ways families are derived during their evolution. In addition, the trees can help clarify the origins of an organism, its evolution pathway, its sequence similarity mutation correlation with other organisms and evolutionary distances [20,53,54,55].

In the present study, *V. cholerae* isolates were examined by sequencing technology to identify them and were recorded according to their 16s rRNA genes. We successfully processed and sequenced all the isolates we wanted to consider [21]. Most of the organisms within our comprehensive tree belonged to *Vibrio cholerae*, which was distributed into three phylogenetic groups. The first easily identified phylogenetic group, group 1, was made up of the majority of the incorporated *V. cholerae* organisms, which, however, constitute the dominant phylogenetic distribution exhibited by this species. Within this group, five of our investigated sequences were resided in two variable positions, namely GenBank isolate accession numbers MK212151.1, MK212153.1, MK212155.1, MK212156.1 and MK212157.1.

Following the group 1 clade, a less predominant group was identified, in which three of our investigated organisms were positioned, namely GenBank isolates MK212152.1, MK212154.1 and MK212158.1. It was inferred from the currently investigated tree that group 2 *V. cholerae* organisms were tilted towards other species within the same genus, namely *Vibrio mimicus* and *Vibrio albensis*. Both the *V. mimicus* and *V. albensis* bacterial organisms exhibited high similarity with some of the incorporated *V. cholerae* species. This finding may be related to the tendency of the group 2 isolates of *Vibrio cholerae* to be positioned in the immediate vicinity to *V. mimicus* and *V. albensis*. This observation indicated a partial tilt of *V. cholerae* in GenBank isolates MK212152.1, MK212154.1 and

MK212158.1 to be diverted from the other related isolates of the same species. However, our investigated *V. cholerae* sequences were not found to reside within the group 3 sequences, which was found to be positioned in another distinct position, away from almost all *V. cholerae*−clustered organisms. However, five of our investigated *Vibrio cholerae* isolates were positioned in group 1 organisms, which did not deviate from the main targeted species. Three of our investigated eight *Vibrio cholerae* isolates had a tendency to reside beside two of the non-*cholerae* species, such as *V. mimicus* and *V. albensis*. This specific positioning might be attributed to the differences in the genetic variation of the currently investigated *Vibrio cholerae* isolates.

Therefore, the eight investigated isolates of *V. cholerae* were positioned in two different phylogenetic positions. The first was represented by the majority of the deposited *V. cholerae* sequences, with no deviation from the main *Vibrio cholerae*. The second position was represented by the less predominant sequences with slight deviation towards some closely related *V. cholerae* sequences. However, the key limiting dividing factor of these three groups came from the pattern of the genetic variations of the 16S rRNA amplicons in the studied eight *Vibrio cholerae* isolates. These variable genetic variabilities may have subdivided these bacterial samples into these previously mentioned phylogenetic groups. When we used *Shewanella* sequences as a highly related outgroup in this tree, no close association was detected in all identified groups with this species, which signifies that the observed 16S rRNA sequences used to construct this tree had provided efficient phylogenetic discrimination among *V. cholerae* isolates at the species level, without being interconnected with the other sequences of highly related organisms.
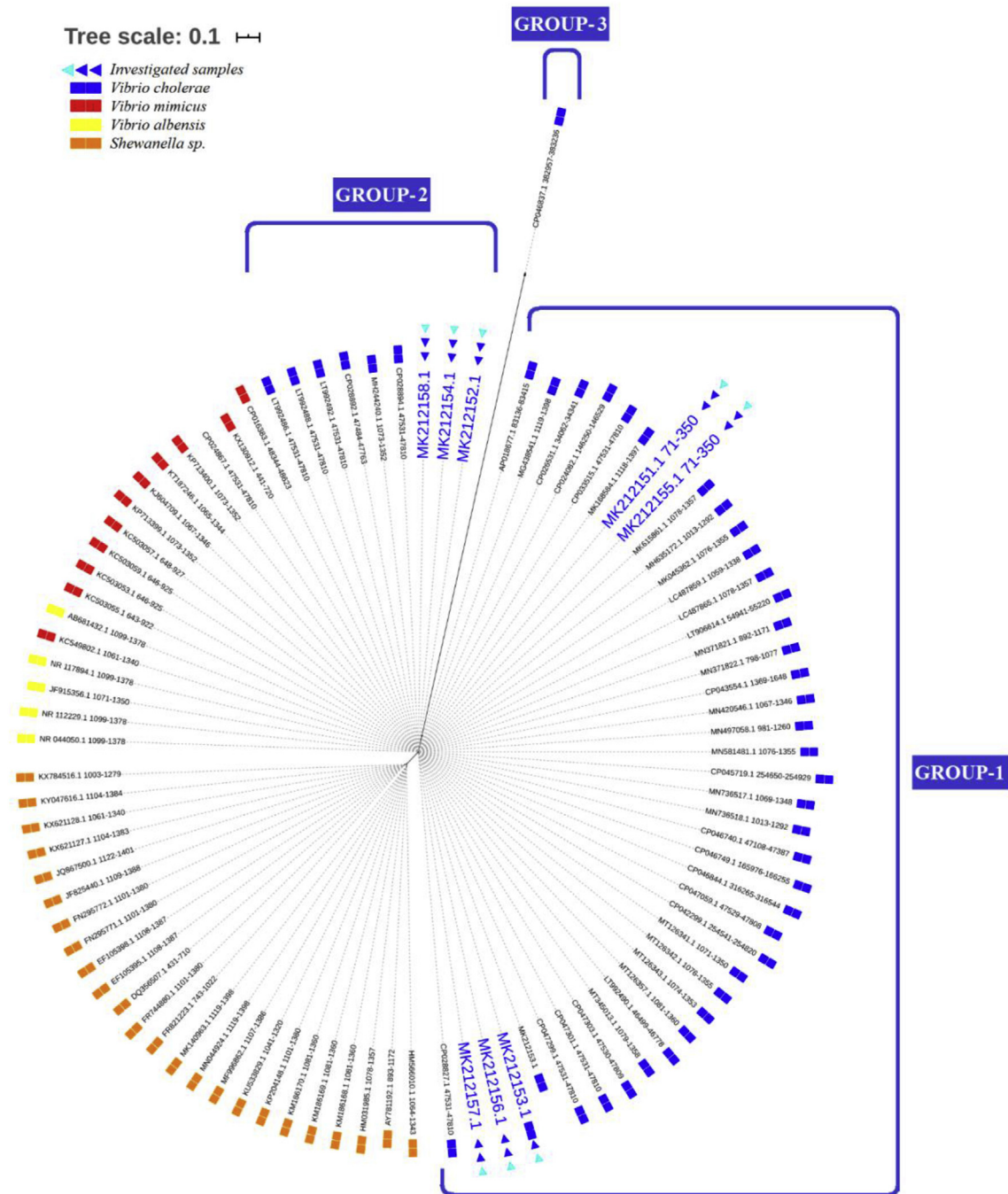
FIG. 10. Neighbour-joining–based phylogenetic tree of genetic variants of 16S rRNA fragment for eight *Vibrio cholerae* local isolates. Numbers refer to virus GenBank accession numbers. Number '0.1' at top left of unrooted tree refers to degree of scale range among comprehensive tree categorized organisms. Different species are highlighted in different colours. *Shewanella* sp. was used as outgroup organism.

Conclusion

Our detection of the 16S rRNA gene provided valuable information about the diagnosis of *Vibrio cholerae* isolates during the

2017 cholera outbreak in Iraq. Not all studied isolates have *rtx* genes. The currently used 16S rRNA–specific primers proved useful in our work to subdivide *V. cholerae* species into two different groups despite their close sequence similarity. This power of high discrimination was added to the original 16S

rRNA−based identity detection ability of a comprehensive tree to accurately identify the precise phylogenetic position of each of the investigated *V. cholerae* species. This in turn further indicates the power of the currently utilized 16S rRNA−based primers to discriminate among *V. cholerae* species. Furthermore, the presented tree adds confirmation regarding the assured identity of *V. cholerae* isolates.

Conflict of interest

None declared.

Acknowledgements

References

[1] Al-Abbassi AM, Ahmed S, Al-Hadithi T. Cholera epidemic in Baghdad during 1999: clinical and bacteriological profile of hospitalized cases. East Mediterr Health J 2005;11:6−13.

[2] Noaman AM, Jumaa LF, Hassan MD. Studying the outbreak of cholera among children in kirkuk central pediatric hospital in 2007. Tikrit J Pure Sci 2011;16:6.

[3] Malik Z, Hasan AB. Epidemiology features of cholera epidemic in Al Hilla city−Babylon province−Iraq, 2015. J Babylon Univ Pure Appl Sci 2018;2:208−16.

[4] Hussein AA. Distribution of *Vibrio* cholera in Iraq during 2017. J Pharm Biol Sci 2018;13:58−61.

[5] Early warning alerts and response network (EWARN), Iraq. EWARN and disease surveillance bulletin. Epidemiological report. Reporting Period May 2017:22−8.

[6] World Health Organization (WHO). Situation report issue 4, 1 april−30 April 2017a. Iraq Humanitarian Emergency; 2017.

[7] World Health Organization (WHO). Situation report issue 5, 1 may−31 may 2017b. Iraq Humanitarian Emergency; 2017.

[8] World Health Organization (WHO). Situation report issue 9, 1−30 November 2017c. Iraq Humanitarian Emergency; 2017.

[9] World Health Organization (WHO). Situation report issue 10, 1−31 December 2017d. Iraq Humanitarian Emergency; 2017.

[10] Al-Hadrawi HAN, Al-Harmoosh RA, Al-Fatlawy HNK. Detection of some virulence factors of clinical *V. cholerae* isolates in Najaf/Iraq. J Pharm Sci Res 2019;11:375−9.

[11] Sack DA, Sack RB, Nair GB, Siddique AK. Cholera Lancet 2004;363: 223−33.

[12] Bag PK, Bhowmik P, Hajra TK, Ramamurthy T, Sarkar P, Majumder M, et al. Putative virulence traits and pathogenicity of *Vibrio cholerae* non-

[13] Chowdhury G, Joshi S, Bhattacharya S, Sekar U, Birajdar B, Bhattacharyya A, et al. Extraintestinal infections caused by non-toxigenic *Vibrio cholerae* non-O1/non-O139. Front Microbiol 2016;7: 144.

[14] Begum K, Ahsan C, Ansaruzzama M, Dutta DK, Ahmad QS, Talukder KA. Toxin(s), other than cholera toxin, produced by environmental non O1 non O139 *Vibrio cholerae*. Cell Mol Immunol 2006;3:115−21.

[15] Kumar P, Jain M, Goel AK, Bhadauria S, Sharma SK, Kamboj DV, et al. A large cholera outbreak due to a new cholera toxin variant of the *Vibrio cholerae* O1 El Tor biotype in Orissa, Eastern India. J Med Microbiol 2009;58(Pt 2):234−8.

[16] Linhartová I, Bumba L, Mašín J, Basler M, Osička R, Kamanová J, et al. RTX proteins: a highly diverse family secreted by a common mechanism. FEMS Microbiol Rev 2010;34:1076−112.

[17] Fullner KJ, Mekalanos JJ. *In vivo* covalent cross-linking of cellular actin by the *Vibrio cholerae* RTX toxin. EMBO J 2000;19:5315. https://doi.org/10.1093/emboj/19.20.5315.

[18] Satchell KJF. Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins of vibrios. Microbiol Spectr 2015;3(3). https://doi.org/10.1128/microbiolspec.VE-0002-2014.

[19] Al-Sa'ady AT. Antibacterial screening for five local medicinal plants against nosocomial pathogens: *Klebsiella pneumoniae* and *Staphylococcus epidermidis*. Eurasia J Biosci 2020;14:553−9.

[20] Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, et al. Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. PLoS Med 2013;10:e1001387.

[21] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis, version 6.0. Mol Biol Evol 2013;30:2725−9.

[22] Chow KH, Ng TK, Yuen KY, Yam WC. Detection of RTX toxin gene in *Vibrio cholerae* by PCR. J Clin Microbiol 2001;39:2594−7.

[23] Sarhan RS, Hashim HO, Al-Shuhaib MBS. The Gly152Val mutation possibly confers resistance to beta-lactam antibiotics in ovine *Staphylococcus aureus* isolates. Open Vet J 2019;9:339−48.

[24] Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li WZ, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 2011;7:539.

[25] Letunic I, Bork P. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 2019;2(W1):W256−9. 47.

[26] Welch RA. RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. Curr Top Microbiol Immunol 2001;257:85−111.

[27] Cheng C, Zhou Y, Kan B, Wang Q, Rui Y. Construction and characterization of a *Vibrio cholerae* serogroup O139 vaccine candidate by genetic engineering. Mol Med Rep 2014;9:2239−44.

[28] Moreno C, Romero J, Espejo RT. Polymorphism in repeated 16S rRNA genes is a common property of type strains and environmental isolates of the genus *Vibrio*. Microbiology 2002;148(Pt 4):1233−9.

[29] González-Escalona N, Romero J, Espejo RT. Polymorphism and gene conversion of the 16S rRNA genes in the multiple rRNA operons of *Vibrio parahaemolyticus*. FEMS Microbiol Lett 2005;246:213−9.

[30] Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol 2008;74: 2461−70.

[31] Hasan AR, Siddique MA, Hasan M, Hossain MA, Rahman MS. 16S rRNA gene sequence based identification of *Vibrio* spp. in shrimp and tilapia hatcheries of Bangladesh. Dhaka Univ J Biol Sci 2017;26: 45−58.

[32] Patel JB. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol Diagn 2001;6:313−21.

O1, non-O139 isolates from surface waters in Kolkata, India. Appl Environ Microbiol 2008;74:5635−44.

[33] Bumba L, Masin J, Fiser R, Sebo P. *Bordetella* adenylate cyclase toxin mobilizes its b2 integrin receptor into lipid rafts to accomplish translocation across target cell membrane in two steps. PLoS Pathog 2010;6:e1000901.

[34] Woo PCY, Ng KHL, Lau SKP, Yip KT, Fung AMY, Leung KW, et al. Usefulness of the MicroSeq 500 16S ribosomal DNA-based identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J Clin Microbiol 2003;41: 1996–2001.

[35] Abbas EC, Al-Saady AT, Al-Khafaji ZAI, Kadhum MJ. Association of IL-17 with hepatitis A virus childhood infection patients less than 10 years old. J Pharm Sci Res 2018;10:1165–6.

[36] Goel AK, Jain M, Kumar P, Jiang SC. Molecular characterization of *Vibrio cholerae* outbreak strains with altered El Tor biotype from southern India. World J Microbiol Biotechnol 2010;26:281–7.

[37] Siriphap A, Leekitcharoenphon P, Kaas RS, Theethakaew C, Aarestrup FM, Sutheinkul O, et al. Characterization and genetic variation of *Vibrio cholerae* isolated from clinical and environmental sources in Thailand. PLoS One 2017;12:e0169324.

[38] Liao F, Mo Z, Chen M, Pang B, Fu X, Xu W, et al. Comparison and evaluation of the molecular typing methods for toxigenic *Vibrio cholerae* in Southwest China. Front Microbiol 2018;9:905.

[39] Boardman BK, Satchell KJ. *Vibrio cholerae* strains with mutations in an atypical type I secretion system accumulate RTX toxin intracellularly. J Bacteriol 2004;186:8137–43.

[40] Lin W, Fullner KJ, Clayton R, Sexton J, Rogers MB, Calia KE, et al. Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. Proc Natl Acad Sci U S A 1999;96:1071–6.

[41] Azman AS, Rudolph KE, Cummings DA, Lessler J. The incubation period of cholera: a systematic review. J Infect 2012;66: 432–8.

[43] Chatterjee R, Sanjay N, Keya C. Identification of a new RTX-like gene cluster in *V. cholerae*. FEMS Microbiol Lett 2008;284:165–71.

[44] Halder M, Mookerjee S, Batabyal P, Palit A. Environmental *Vibrio cholerae* non O1/non O139 from the Gangetic delta: a diarrhoeal disease purview. Int J Environ Health Res 2017;27:241–51.

[45] Kumar P, Peter WA, Thomas S. Rapid detection of virulence-associated genes in environmental strains of *Vibrio cholerae* by multiplex PCR. Curr Microbiol 2010;60:199–202.

[46] Xu MJ, Wu JR, Chen LM. Virulence, antimicrobial and heavy metal tolerance, and genetic diversity of *Vibrio cholerae* recovered from commonly consumed freshwater fish. Environ Sci Pollut Res 2019;26: 27338–52.

[47] Sedas VT. Influence of environmental factors on the presence of *Vibrio cholerae* in the marine environment: a climate link. J Infect Dev Ctries 2007;1:224–41.

[48] Paz S. Impact of temperature variability on cholera incidence in southeastern Africa, 1971–2006. Ecohealth 2009;6:340–5.

[49] Constantin de Magny G, Colwell RR. Cholera and climate: a demonstrated relationship. Trans Am Clin Climatol Assoc 2009;120:119–28.

[50] Al-Sa'ady AT. Detection of vancomycin resistance in multidrug-resistant *Enterococcus faecalis* isolated from burn infections. Drug Invention Today 2019;11:2984–9.

[51] Karim FA, Darwish LAA. Threatening reoccurring infection of cholera in Kerbala province. Sci J Med Res 2018;2:124–9.

[52] Natale DA, Shankavaram UT, Galperin MY, Wolf YI, Aravind L, Koonin EV. Towards understanding the first genome sequence of a crenarchaeon by genome annotation using clusters of orthologous groups of proteins (COGs). Genome Biol 2000;1(5). RESEARCH0009.

[53] Santamaría R, Therón R. Treevolution: visual analysis of phylogenetic trees. Bioinformatics 2009;25:1970–1.

[54] Soares A, Râbeloa R, Delbemb A. Optimization based on phylogram analysis. Exp Syst Appl 2017;78:32–50.

[55] Al-Fatlawy HNK, Aldahhan HA, Alsaadi AH. Phylogenetic of ERIC-DNA fingerprinting and new sequencing of *Aeromonas* species and *V. cholerae* DNA. Am J Appl Sci 2017;14:955–64.