REVIEW ARTICLE

Phylogeny of *Vibrio cholerae* Isolates from Patients with Cholera Disease in Babylon Province

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ABSTRACT

In the present study, a total of 35 stool samples were collected in the Central Health Laboratory of Babylon Province from patients presenting invasive cholera disease. The period of collection was from November 2017 to December 2018. Identification of *Vibrio cholerae* species was carried out by conventional methods, biochemical tests, diagnostic kits and then confirmed by PCR-based assay targeting *the ompW* gene. The results of this study reported that O1 serogroups were the predominant serogroup among all clinical samples with a high rate of 94.3% (N = 33), while only two isolates of non-O1/non-O139 (NAG) (5.7%) were documented as a causative agent to cholera or cholera-like disease. The phylogenetic relationship among all 35 studied strains elucidated by using polymerase chain reaction (PCR)-based fingerprinting assay (ISSR-PCR). The results of this assay showed grouping of Inaba strains into different clusters indicating that these strains were genetically diverse. Furthermore, *V. cholerae* El Tor O1 Ogawa strain (OG1) was closely related to strains of Inaba serotype. In contrast, NAG strains (NAG1 and NAG2) were not genetically similar to any of Inaba or Ogawa strains indicating different clone origin.

Keywords: Babylon Province, Cholera Disease, *Vibrio cholerae.*

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INTRODUCTION

Vibrio cholerae is oxidase-positive, gram-negative curved bacilli that are motile by the presence of polar flagellum. They are non-capsulated, non–spore-forming and they can grow under aerobic or anaerobic conditions.[1] *V. cholerae* is widely distributed in aquatic environments such as estuarine, freshwater, and marine. In addition, *V. cholerae* has been associated with outbreaks of the most feared epidemic diarrheal disease, called cholera.[2,3] Cholera is a severe diarrheal illness transmitted by contaminated water and food.[4] This disease is responsible for sporadic epidemic cases, and seven pandemics have been reported that resulted in thousands of deaths and major changes in socio-economic situations worldwide.[5,6] In Iraq, Cholera is endemic with a cyclic trend every four to five years and clustered mainly in the middle and south provinces during the last outbreaks. Meanwhile, Babylon province is one of the most effected to this disease after Baghdad during the last 2015 and 2017 outbreaks.[7-9] Regarding variations in the structure of O-antigen, there are over 200 serogroups of *V. cholerae*, where only O1 and O139 were found to be associated with a pandemic, endemic, and epidemic cholera.[10] In addition to these serogroups, non-O1/non-O139 strains were reported from several countries as a causative agent for sporadic outbreaks of diarrhea, cholera-like disease.[3] With regard to phenotypic features and the presence of O antigens in *V. cholerae* O1, there are two distinct biotypes, called classic and El Tor, and three serotypes for each biotype, called Ogawa, Inaba, and Hikojima.[11]

Molecular fingerprinting methods have discriminatory power among epidemiologically non-related strains and in the determination of closely related strains.[12,13]

Indeed*, several* fingerprinting methods used for typing of *V. cholerae* such as pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD)-PCR, amplified fragment length polymorphism (AFLP), enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR, variable number tandem repeats (VNTRs), multi-locus sequence typing (MLST), multi-locus variable tandem repeat analysis (MLVA), repetitive extragenic palindromic (REP)-PCR and Ribotyping.[14-16] Recently, inter simple sequence repeat (ISSR)-PCR used as a novel assay of genotyping of *V. cholerae*, where this assay is an *efficient tool to* reveal the phylogenetic relationships among *V. cholerae* strains.[17]

MATERIALS AND METHODS

Thirty-Five stool samples were obtained from patients suspected with cholera disease in the Central Health Laboratory of Babylon province through the period from November 2017 to

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December 2018. The suspected stool samples were transported to the Central Health Laboratory of Babylon province from different hospitals in Babylon province by using the Cary Blair medium. After the recording of patient information, the obtained stool samples were cultured on Alkaline peptone water (APW) to enrich *V. cholera* and then isolated on selective and non-selective media for further identification processes.[18] *V. cholerae* pathogens were identified by conventional cultural methods, Gram stain, biochemical tests, diagnostic kits (API 20 E Kit and VITEK 2 Compact System), biotyping (Blood hemolysis, Polymyxin-B sensitivity, and Voges-Proskauer test) and serotyping according to Kay *et al.,*[19] and kit manufacturer's instructions. Then, the confirmatory identification was done by using uniplex PCR method.

Molecular methods

DNA extraction

Genomic DNA of studied *V. cholerae* strains was extracted by using a Genomic DNA purification kit supplied from Geneaid company, UK.

Primer design

The oligonucleotide primers used in this study were obtained from previous studies, as shown in Table 1.

Uniplex PCR method

This assay used to identify *V. cholerae* by using *ompW* specific primers as shown in Table 1. This assay was performed according to Nandi *et al.,*[20] with minor modification regarding PCR mixtures and conditions. PCR amplification of DNA was carried out in final reaction mixture volume of 25 µl and within 30 cycles. Products of PCR amplification were electrophoresed by 1.5% agarose gel and then visualized under UV-transilluminator after staining with ethidium bromide. PCR mixtures and PCR conditions of this assay were summarized in Table 2.

Fingerprinting assay (ISSR-PCR)

In this study, the Inter Simple Sequence Repeat-PCR (ISSR-PCR) assay used as a fingerprinting assay for the elucidation of the phylogenetic relationship among studied *V. cholerae* isolates. This assay was carried out according to Kumar *et al.,*[17] protocol with few modifications in PCR mixtures. By this assay, eight arbitrarily designed ISSR primers (listed in Table 1) were used to amplify different interspersed genomic regions between simple sequence repeats. ISSR primers contained repetitive sequences (five di- and three trinucleotide repeats) complementary to microsatellite regions in *V. cholerae* genome. ISSR-PCR was performed as a single-primer PCR amplification adjusted to 25µL final mixture volume through 35 cycles. Table 3 summarized both PCR mixtures and conditions used in this assay. The PCR products were separated on 2.5%

Table 1: Primers sequences of this study with their amplicon size (bp) and reference.

Gene		*Sequence*	*Product size bp*	*Reference*
ompW	Sense	5'- CACCAAGAAGGTGACTTTATTGTG-3'	304	(20)
	Antisense	5'- GGTTTGTCGAATTAGCTTCACC -3'		
ISSR-Primers	(GA)8T	5'-GAGAGAGAGAGAGAGAT-3'		(17)
	(CA)7C	5'-GCTAGTGCTCACACACACACACAC-3'		
	C(GA)7	5'-GACGATACGAGAGAGAGAGAGA-3'		
	(GA)8C	5'-GAGAGAGAGAGAGAGACGG-3'		
	(ATG)4GA	5'-ATGATGATGATGGACT-3.'		
	GC(GCC)4	5'-TGAGCGCCGCCGCCGCC-3'		
	TA(CAG)4	5'-AAATACAGCAGCAGCAG-3'		
	T(GA)8	5'-TGTAATGAGAGAGAGAGAGAGA-3'		

Table 2: Uniplex PCR mixtures and conditions for identification of the *ompW* gene.

PCR mixtures		*PCR conditions*		
Contents	*Volume*	*Type of cycle*	*Condition*	*No. of cycles*
Master mix	12.5 µl	Initialization	95 °C for 5 min	1
Forward primer	2.5 µl	Denaturation	94 °C for 1 min	30
Reverse primer	2.5 µl	Annealing	58 °C for 1 min	
Template DNA	3 µl	Extension	72 °C for 1 min	
Nuclease-free water	4.5 µl	Final Extension	72 °C for 10 min	1

Table 3: ISSR-PCR mixtures and conditions.

PCR mixtures		*PCR conditions*		
Contents	*Volume*	*Type of cycle*	*Condition*	*No. of cycles*
Master mix	12.5 µL	Initialization	94 °C for 3 min	1
Primer	1 µL	Denaturation	94 °C for 1 min	35
Template DNA	3 µL	Annealing	50 °C for 30 sec	
Nuclase-free water	8.5 µL	Extension	72 °C for 2 min	
		Final extension	72 °C for 10 min	1

agarose gel stained with ethidium bromide for 2 hours at 80v and visualized by UV illuminator. After image capturing, gel images were processed by Image lab software (Version 6.0.1, Bio-Rad Laboratories, Inc.) and prepared for further analysis. All further analysis steps were carried out by BioNumerics software (Version 7.6.3) such as automatic band detection, sizing, scoring the data and finally building an UPGMA-based phylogenetic tree.

Statistical analysis

Data were processed by using statistical program social science (SPSS 22). In this study, microbial isolation results were analyzed by using Chi-Square and Binomial test at a 5% level of significance.[21]

RESULTS AND DISCUSSION

Isolation and Identification of *Vibrio cholerae*

A total of 35 stool samples were obtained from patients who are presenting invasive cholera disease from the Central Health Laboratory of Babylon Province during the period from November 2017 to December 2018. *Vibrio cholerae* pathogens were identified by conventional methods and confirmed by using Molecular methods. Regarding Biotyping diagnosis, the results of this study indicated that El Tor biotype had prevailed in all clinical isolates of *V. cholerae* with a high rate (100%) rather than the Classic biotype that showed a negative result for all clinical samples as shown in Table 4.

With regard to serogrouping and serotyping diagnosis of *V. cholerae* isolates, the results revealed that O1 serogroups were the predominant serogroup among all clinical samples with a high rate 94.3% (N =33), while only two isolates of non-O1 (NAG) (5.7%) documented as a causative agent to cholera or cholera-like disease. In contrast, the results of the study showed the negative result to identify O139 as shown in Table 4. With regard to identification of serotypes, Inaba serotype had prevailed in all O1 serogroup of *V. cholerae* with a high percentage 97% (N = 32), while only one Ogawa serotype (3%) was reported as a causative agent for cholera disease in Babylon province as shown in Table 4. To differentiate among *V. cholerae* species and other very close diarrhea-caused bacteria such as *Vibrio mimicus, Vibrio parahaemolyticus* and *Aeromonas hydrophila*, Uniplex PCR method were used depending on detection of *ompW* gene encoding the outer membrane protein by using *ompW*-specific primer to *V. cholerae.* This method generates reproducible profiles of *V. cholerae*-specific (304 bp) amplicons as shown in Figure 1. These results were statistically analyzed and revealed that this is a significant difference among percentages of serogroups (O1, O139, NAG), where the p-value is <0.00001 ($p<0.05$). Moreover, this is a significant difference among *V. cholerae* serotypes (Inaba 97% and 3% Ogawa), where p-value is <0.00001 ($p<0.05$), as shown in Table 4.

As described above, the results of this study documented that outbreak of cholera disease in Babylon Province occurred at the period of study with 100 Positivity of *V. cholerae,* these results bring into line with other studies[7,9,22] who noted the occurrence of cholera outbreak in Babylon Province during the past years. In addition, the results of this study revealed that El Tor biotype, O1 serogroup, and Inaba serotype were the predominant type of *V. cholerae* classification in Babylon province. These results were in agreement with other studies in Babylon and other Provinces of Iraq,[9,23-26] while these results were in disagreement with other studies inside Babylon Province[27] or in all Iraqi Provinces[28] who documented that *V. cholerae* El Tor O1 Ogawa was the most common cause of cholera in Babylon and Iraq during the years 1998 and 1999 rather than *V. cholerae* El Tor O1 Inaba. The differences and similarities in this study with others could be related with climate change during the past years in Iraq, where Constantin de Magny and Colwell[29] mentioned that climate

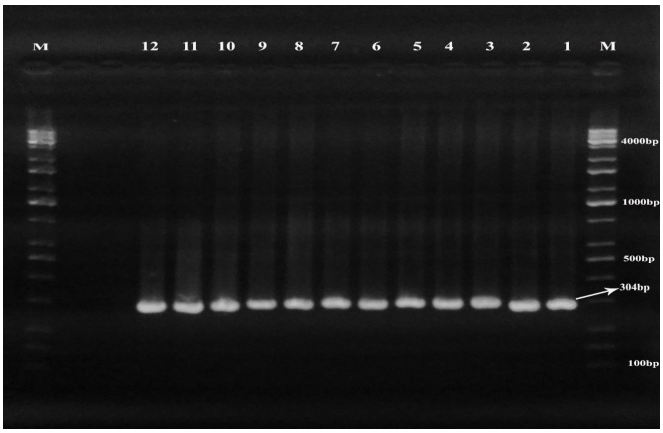

Figure 1: Agarose gel electrophoresis of PCR products obtained with bacterial strains using an *ompW*-specific primer. The bacterial strains used were *V. cholerae* O1 El Tor Ogawa (lane 1-2), *V. cholerae* O1 El Tor Inaba (3-10) and non-O1/non-O139 NAG (lanes 11-12). Lane M represents 100bp universal DNA ladder, white arrow represents PCR amplicon.

Table 4: Distribution of *Vibrio cholerae* serogroups, biotypes and serotypes isolated from patients with cholera disease in Babylon province.

	El Tor No. (%)			*Classic No. (%)*			
Serogroups	*Inaba*	*Ogawa*	*Total*	*Inaba*	*Ogawa*	*Total*	*p value*
O1	32 (97)	1 (3)	33 (94.3)	0 (0)	0 (0)	0 (0)	a- <0.00001 b- <0.00001
O139	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
NAG	2 (5.7)						
Total	35 (100)						

* represents a significant difference at $p<0.05$. NAG, non-O1/non-O139. Litters (a and b) represent type of the statistical analysis between a-serogroups (O1, O139, Nag), b- serotypes (Inaba and Ogawa).

and environmental changes may result in modification of the population size of *V. cholerae* in the environment and will influence the emergence of cholera disease in human populations. Meanwhile, the quality and availability of drinking water can be changed in unstructured conditions, such as war situation,[30] so the environment may have converted to be more suitable for *V. cholerae* El Tor O1 Inaba in contrast to *V. cholerae* El Tor O1 Ogawa.

Also, another study by Siriphap *et al.,*[31] was in disagreement with the result of this study, where they reported that the classical biotype causing cholera outbreaks in Thailand as predominant biotype. The predominance of El Tor biotype in Iraq could be related to fact that El Tor biotype known to tolerate a wider range of environmental conditions and also is thought to persist longer in the harsh environment better than the classical biotype, for that reason El Tor O1 biotype has now virtually displaced the classical biotype completely throughout the world.[11,32,33] The species-specific identification of *V. cholerae* strains depends on an outer membrane protein (OmpW) encoding gene *ompW.* The presence of this gene in *V. cholerae* strains is related to the fact that its nucleotide sequence remained practically unchanged among the different strains of *V. cholerae* and considered as a highly suitable genetic marker for this organism.[20] For that reason, *ompW*-specific primer used in uniplex and multiplex PCR methods of this study with 304bp amplicon, these results were in disagreement with Mehrabadi *et al.,*[34] who used 588bp PCR amplicon of *ompW* to differentiate between *V. cholerae* and other Gram-negative bacteria.

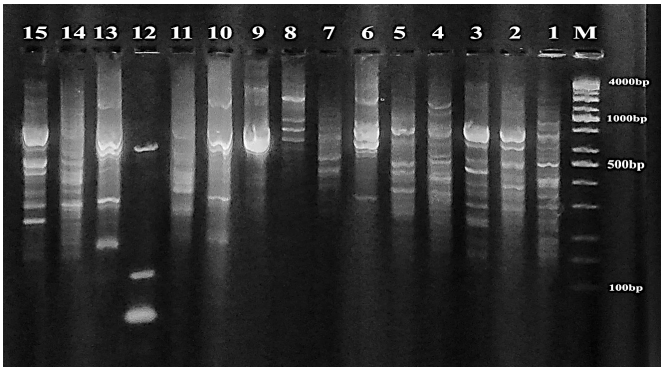

Figure 2: Genomic fingerprints -derived profile by ISSR-PCR data for the studied bacterial strains. Lane M, 100bp universal DNA ladder; lanes1-15, IN1-IN15 bacterial strains of *Vibrio cholerae* O1 Inaba. This profile generated by (CA)7C primer run on 2.5% agarose gel.

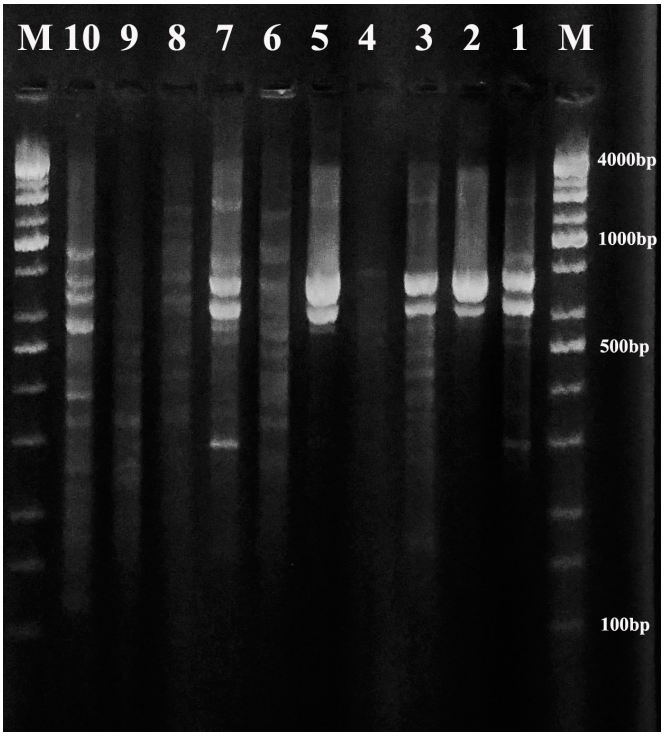

Figure 3: Genomic fingerprints-derived profile by ISSR-PCR data for the studied bacterial strains. Lane M, 100bp universal DNA ladder; lanes1-10, IN16-IN25 bacterial strains of *Vibrio cholerae* O1 Inaba. This profile generated by (CA)7C primer run on 2.5% agarose gel.

Fingerprinting

In the present study, PCR-based fingerprinting assay (inter simple sequence repeat-PCR [ISSR-PCR]) was used to elucidate the phylogenetic relationship among all 35 studied *V. cholerae* isolates (Inaba, Ogawa and NAG). By this assay, eight ISSR primers were used to analyze *V. cholerae* isolates. Basically, different genomic regions that are interspersed between closely spaced simple sequence repeats (SSR) were amplified by these primers, as shown in Figures 2, 3, and 4 that documented ISSR-PCR profile of (CA)7C primer.

The results of this assay for all used eight primers revealed production a total of 232 bands ranging from 110 to 3500 bp. Form these bands; a phylogenetic cladogram was generated based on the cluster analysis by using the unweighted pair group with arithmetic averages (UPGMA) method, as visualized in Figure 5.

The results of this study showed grouping of *V. cholerae* El Tor O1 Inaba strains into different clusters indicating that these strains were genetically diverse. For example, the strains IN23, IN21, IN11, IN7, IN2 and IN32 grouped into one cluster with different clades suggesting their single clonal origin. Similarly, the strains IN18, IN16, IN6, IN22, IN13, IN31, and IN15 represented one cluster with different clades. Moreover, the clonal nature of the strains IN30, 28, IN29, IN27, IN25,

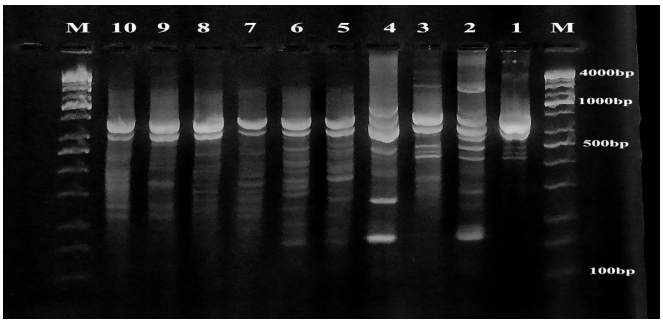

Figure 4: Genomic fingerprints -derived profile by ISSR-PCR data for the studied bacterial strains. Lane M, 100bp universal DNA ladder; lanes1-10, OG1, NAG1, IN26, NAG2, IN27-IN32 of *Vibrio cholerae* O1 Ogawa, Inaba and Non O1 (NAG) serotypes. This profile generated by (CA)7C primer run on 2.5% agarose gel.

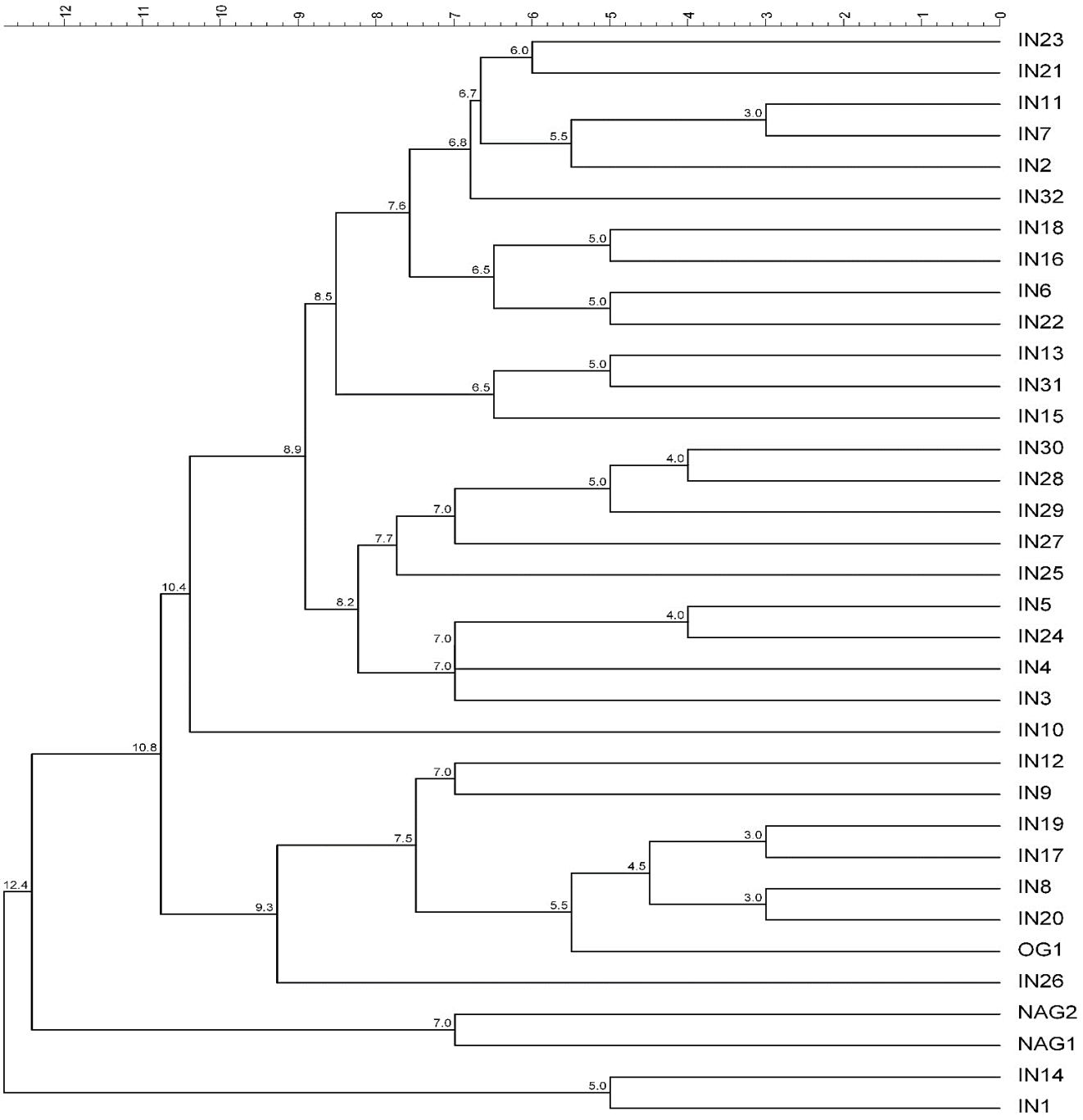

Figure 5: The ISSR-PCR-derived cladogram representing the relationship among 35 strains of *Vibrio cholerae* relating to Inaba, Ogawa and non-O1/non-O139 (Nag) serogroups. Bar represents the distance values.

IN5, IN24, IN4, and IN3 also indicated the presence of one cluster with different clades. Regarding the last mentioned strains, very close genetic similarity was noted among each cluster. In addition, this genetic similarity noted again among another cluster of the strains IN12, IN9, IN19, IN17, IN8, and IN20. On the other hand, four strains of *V. cholerae* El Tor O1 Inaba (IN10 IN26 IN14 and IN1) were not clustered in any of the last-mentioned groups. Among these strains, the clade (IN14 and IN1) was distinctly different, suggesting new clone origin. Furthermore, the results of this study showed that *V. cholerae* El Tor O1 Ogawa strain (OG1) was closely related to strains of Inaba serotype in the clade (IN19, IN17, IN8 and IN20) and they have shared an ancestor. Regarding *V. cholerae* Non-O1/Non139 NAG strains (NAG1 and NAG2), results of this study showed that these strains were not genetically similar to any of the *V. cholerae* El Tor O1 Inaba or Ogawa strains, indicating that *V. cholerae* Non-O1/Non139 NAG strains had different clone origin.

Interestingly, the genetic diversity among *V. cholerae* El Tor O1 Inaba strains was in agreement with other studies[17,35-37]

who reported grouping of *V. cholerae* El Tor O1 strains into different clusters by using other fingerprinting methods in Iraq and other countries. In contrast, the observation of Pichel *et al*.[38] was in disagreement with results of this study, where they reported that strains of *V. cholerae* O1 belong to a single clonal cluster in Argentina. The genetic diversity among these strains may be related to gene deletion, duplication, within-patient mutations or the high rates of horizontal gene transfer mechanisms.[39,40,41,42]

Furthermore, the close phylogenetic relationship between the *V. cholerae* El Tor O1 Ogawa and Inaba serotypes that noted in this study agreed with other studies,[43,44] who reported the emergence of *V. cholerae* O1 Inaba from *V. cholerae* O1 Ogawa stains. Moreover, Colwell *et al.,*[45] documented the interconversion between Ogawa and Inaba serotypes of *V. cholerae* O1 stains, and this switching between *V. cholerae* O1 serotypes could be related to several changes regarding the genetic form of the *wbtT* gene that responsible to the determination of Ogawa specificity.[46]

Meanwhile, the result of this study noted that *V. cholerae* Non-O1/Non139 NAG strains were not genetically related to *V. cholerae* El Tor O1 Inaba or Ogawa strains, while Shuan Ju Teh *et al.,* 15 disagreed with this results when they mentioned the close genetic relationship between *V. cholerae* non-O1/non-O139 strains and O1 strains in Malaysia. However, the results of Singh *et al*.[47] agreed on this study when they reported that *V. cholerae* non-O1, non-O139 strains were more diverse and not related to O1 strains. These differences among studies may be related to the natural selection mechanisms among *V. cholerae* non-O1, non-O139 strains, where these mechanisms were different according to the environmental source. In addition, the highly diverse and the distinct genomic patterns of *V. cholerae* non-O1, non-O139 strains could be caused by genetic mutations or gene recombination.[48]

In toto, the data of this study have public-health implications, where the highly diversity and the emergence of a new clone of *V. cholerae* in Babylon province calls attention for the epidemic tracing of outbreak strains and the recognition of strains with new clones that could protrude in the future, that in turn very important for understanding the evolution of *V. cholerae* and cholera disease.

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