

Phylogenetic of ERIC-DNA Fingerprinting and New Sequencing of *Aeromonas* Species and *V. Cholerae* DNA

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Abstract: The current study included 44 isolate of *A. hydrophila*, *A. sobria* and *V. cholerae* and other bacteria isolated from stool samples and environmental samples (Kufa river water and hospital environmental samples). ERIC DNA Fingerprinting with ERIC primers pairs generated distinct amplification bands ranging in size from (87 bp to 8000 kb). The 44 isolates produced 93 different patterns by ERIC DNA fingerprinting. The fingerprinting patterns of the isolates were constructed using cluster analysis the UPGMA (group method) using average linkages Number of different bands (Similarity coefficient), 1% tolerance. The PCR method of gene (*16SrDNA* and *16SrRNA*) were the best methods for diagnosis, which has led to isolate and diagnosis of *A. hydrophila* *A. sobria* and *V. cholerae* are distributed as clinical isolates of *A. hydrophila* *A. sobria* were diarrhea samples. While, the environmental isolates were isolate of *V. cholerae* from Kufa river water. Sequencing technology is used to diagnosis of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates were examined by (*16SrDNA*, *16SrRNA*) genes. Recorded the new isolates in Nucleotide/Blast and recorded as the first sequencing in Gene-Bank/NCBI, DDBJ and ENA (INSDC). Each sequence have Accession number (No.: Gene bank: LC194875 *Aeromonas sobria*-Hnk1, Gene bank: LC194876 *Aeromonas hydrophila*-Hnk2, Gene bank: LC194877 *Vibrio cholerae*-Hnk3) this is the first study in Iraq for discovery of new isolates by new sequences. The frequency of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates in Najaf were higher among clinical and environmental isolates. The ERIC band pattern is an adequate tool for epidemiological investigations of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates.

Keywords: ERIC, *A. Hydrophila*, *A. Sobria*, *V. Cholerae*, Phylogenetic, Sequencing

Introduction

The Gram-negative bacilli *Aeromonas hydrophila* and *Aeromonas sobria* are species of the genus *Aeromonas*, which belongs to the family Aeromonadaceae that received increasing attention opportunistic pathogen s because of its association with both dysenteric diarrheal and extra intestinal infections in human disease especially in children and persons with impaired immune system (Naharro *et al.*, 2009; Uche and Johnkennedy, 2014). *Aeromonas* bacteria are linked to two types of gastroenteritis, the first type is a disease similar to cholera which causes rice-watery diarrhea and the other type of disease is dysenteric gastroenteritis that causes loose stools filled with blood and mucus, while dysenteric

gastroenteritis is the most severe out of the two types and distributed of *A. hydrophila* is widely in fresh and salt water also frequently found in chlorinated and non-chlorinated drinking water (Galindo and Chopra, 2007).

And other hand, Cholera is an acute diarrheal infection caused by ingestion of food or water contaminated with the bacterium *Vibrio cholera* that belongs to genus vibrio, family Vibrionaceae (WHO, 2016).

Genetic diversity studies on *Aeromonas spp.* have received a little attention in Iraq. The present study is the first study in Najaf/Iraq and carried out to achieve the following objectives 1. Isolation of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates from diarrheal samples and environmental samples and identification

by diagnosis genes (*16SrDNA*, *16SrRNA*) during PCR technique. 2- Phylogenetic analysis by ERIC DNA fingerprinting of *A. hydrophila*, *A. sobria*, *V. cholerae* isolates and other bacteria. 3- Sequencing technology is used in this study. 4- Recorded new Iraqi sequences in Gene-Bank/NCBI/USA.

Material and Methods

Samples Collection

Diarrheal samples were 272 from patients suffering of diarrhea infection in AL-Main Health Laboratory of Najaf governorate during the period (April 2016 to November 2016). While, 34 environmental samples were involve three different of environmental regions (Kufa river water). *A. hydrophila* isolates were diagnosed by four methods as (Culture, biochemical tests, Vitek@2GN/ID cards system and Polymerase Chain Reaction (PCR) methods). Most characteristics of *A. hydrophila*, *A. sobria* and *V. cholerae* bacteria were examine dofgam's stain bacteria, the microscopic properties (Jawetz *et al.*, 2016). Culturally, colonies characteristics of isolates were recorded on the specific media for primary identification of *A. hydrophila* *A. sobria* and *V. cholerae* (Collee *et al.*, 1996) and ThioSulphate Citrate Bile Salt Sucrose Agar (TCBS) (Henry, 1996), Alkaline Peptone Water Medium (APW) (Gomez-Gil and Roque, 2006) and *Aeromonas* Isolation Medium Base (Moyer, 1987).

Biochemically tests were in dole tests, oxidase test and simmone citrate test (MaccFadin, 2000).

VITEK@2 GN ID Card System

The identified *Aeromonas* ssp and *V. cholerae* isolates were confirmed with the automated VITEK@2 compact system by using GN ID cards. The GN ID card is based on established biochemical (64 reaction) methods and newly developed substrates, measuring various metabolic activities (BioMérieux Company/http://www.bioMérieux.com),

Genomic DNA Extraction Bacteria Chromosomal DNA (Promega/USA)

Extraction Chromosomal DNA is 44 isolates of (34 *A. hydrophila* 2 *A. sobria*, 3 *V. cholerae*, 1 *E. coli* O 157, 1 *E. coli*, 1 *Pseudomonas aeruginosa*, 1 *Enterobacter complex* and 1 *Acinetobacter manniuii*) bacteria. The wizard genomic DNA purification kit is designed for extraction of DNA from bacteria. Extraction of genomic DNA was performed as follow:

- Added 1 mL of an overnight culture to a 1.5 mL microcentrifuge tube
- Used centrifuge the growth at 14,000×g for 2 min to pellet the cells and remove the supernatant

- Added 600 µL of Nuclei Lysis solution. Gently pipet until the cells were re-suspended
- Incubated at 80°C in water bath for 5 min to lyse the cells; then cool it at room temperature
- Added 3 µL of RNase solution to the cell lysate. Invert the tube 2-5 times to mix
- Incubated at 37°C for 45 min. Cooling the sample at room temperature
- Added 200 µL of protein precipitation solution to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 sec to mix the protein precipitation solution with the cell lysate
- Incubated the sample on ice for 5 min
- Centrifuge at 14,000×g for 3 min
- Transferred the supernatant containing DNA to a clean 1.5 mL microcentrifuge tube containing 600 µL of room temperature isopropanol.
- Gently mixing by inversion until the thread-like strands of DNA form a visible mass
- Centrifuge at 14,000×g for 2 min
- Carefully pouring off the supernatant and draining the tube on clean absorbent paper. Adding 600 µL of 70% ethanol at room temperature and gently inverting the tube several times to wash the DNA pellet
- Centrifuge at 13,000-16,000×g for 2 min. Carefully aspirate the ethanol
- Draining the tube on clean absorbent paper and allow the pellet to air-dry for (10-15) min
- Added 100 µL of DNA rehydration solution to the tube and rehydrate the DNA by incubating at 65°C for 1 h Periodically mixing the solution by gently tapping the tube. Alternatively, rehydrating the DNA by incubate the solution overnight at room temperature or at 4°C
- Store the DNA at 2-8°C

PCR Amplification

PCR technique has been amplify genes of *16SrDNA*, *16SrRNA* with ERIC1, ERIC2 primers with genomic DNA of all isolates *A. hydrophila* *A. sobria* and *V. cholerae* and other bacteria. The wizard genomic DNA purification kit is designed for extraction of DNA *A. hydrophila* *A. sobria* and *V. cholerae* and other bacteria. Gel electrophoresis was used for detection of DNA by UV transilluminator (Sambrook and Russell, 2013). The PCR assay was performed to detect the (16S rRNA, *16SrDNA*) gene for confirmation the identification of *A. hydrophila* *A. sobria* and *V. cholerae* and other bacteria. These primers synthesized by Alpha DNA company, Canada Program of PCR.

PCR products and the DNA marker are resolved by electrophoresis on (1.5%) agarose gel shown in Table 1.

Clonal Analysis by ERIC DNA Fingerprinting

A: ERIC-PCR Assay of *A. Hydrophila* and *A. Sobria*, *V. Cholerae* and Other Bacteria

According to Rathinasamy *et al.* (2014), the assessing genotypic distinctions in *Aeromonas hydrophila*, *Aeromonas sobria*, *Vibrio cholera* and *E. coli* O157 isolates, direct PCR examination was carried out to these isolates. One set of primers were used specifying. The reaction was carried out by using a 50 μ L mixture including (25) μ L GoTaq® Green Polymerase Mix, (5) μ L for each ERIC 1 and ERIC2 separately, (8) μ L of genomic DNA and the volume was completed with nuclease free water.

The PCR was performed with a Biometra, professional thermal cycler under the following conditions.

The PCR products were loaded with gel electrophoresis after mixing with loading dye. The electrophoresis done with Biometra gel electrophoreses in 1.5% agarose gels and photographed on gel documentation system.

B: Bioinformatics' Analysis for ERIC DNA Fingerprinting

Computer analysis of ERIC DNA fingerprinting was carried out using Bio Numerics Gel-Compar II version 7.6. Applied Maths. Similarity between fingerprints were calculated with the Dice coefficient. Cluster analysis was performed using the unweighted pair-group method.

Gene Sequencing and Analysis

Sequencing of diagnosis genes for *Aeromonas hydrophila*, *Aeromonas sobria* and *Vibrio cholera* (*16SrRNA*, *16SrDNA* genes) were performed by Macrogen's sequencing service, Sequencing Technology in Korea <http://dna.macrogen.com>.

Sequence Analysis Software Programs; blast online program (www.ncbi.nlm.nih.gov). Basic Local Alignment Search Tool (BLAST) program is available at the National Center Biotechnology Information (NCBI) online at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> for *Aeromonas hydrophila* and *Aeromonas sobria* and *Vibrio cholera* isolates in this study.

Bioinformatics

Bioinformatic and biostatistical service technology has been advancing the field of medical and ecological research and diagnostics in Next-Generation Sequencing (NGS). Bioinformatic specialized in life science and setting new standards for high level services of data analysis including high expertise in next-generation-sequencing (Ghatak *et al.*, 2016).

Statistical Analysis

Statistical analysis by SPSS computing program (version 24) for the analysis (<https://www.ibm.com/marketplace/cloud/statistical-analysis2015>).

Results

Identification of *Aeromonas spp.* and *V. Cholerae*.

During the study period were collected 272 clinical samples from diarrhea cases and, 34 environmental samples from Kufa river water and hospital environmental samples Identification of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates is depended on initial identification the colonial morphology, microscopically and biochemical tests. The colonies of *A. hydrophila* *A. sobria* and *V. cholerae* are grown on culture media once revealed the typical characteristics, on blood agar *A. hydrophila*, *A. sobria* and *V. cholerae* produces smooth, convex, rounded and β -hemolytic colonies and pale white to grey color, but colonies were green with black center *Aeromonas* isolates on *Aeromonas* media and *V. cholerae* on Chromo agar is light blue colonies as show in (Fig. 1). Biochemically tests were positive results for indole tests, oxidase test and simmone citrate test.

VITEK@2 GN ID Card System

The identification was contained biochemical tests. The results demonstrate that isolates of *A. hydrophila* *A. sobria* and *V. cholerae* isolates were confirmed with ID message confidence level ranging excellent (Probability percentage from 94 to 99.7%). The 44 isolates of (34 *A. hydrophila* 2 *A. sobria*, 3 *V. cholerae*, 1 *E. coli* O 157, 1 *E. coli*, 1 *Pseudomonas aeruginosa*, 1 *Enterobacter complex* and 1 *Acinetobacter manniuii*.) of isolates on MacConkey agar.

Molecular Identification

PCR technique has been amplify genes of *16SrDNA* and *16SrRNA* genes with genomic DNA of all isolates *A. hydrophila*, *A. sobria* and *V. cholerae*. The results of all isolates diagnosis by PCR technique for detection both *16SrDNA* and *16SrRNA* clarify isolates of *A. hydrophila*, *A. sobria* and *V. cholerae* producers carrying *16SrDNA* and *16SrRNA* genes, as show in (Fig. 2).

Phylogenetic of ERIC-DNA Fingerprinting

ERIC DNA Fingerprinting with ERIC primers generated amplification bands ranging in size (87 bp to 8000 kb). The 44 isolates of (34 *A. hydrophila* 2 *A. sobria*, 3 *V. cholerae*, 1 *E. coli* O 157, 1 *E. coli*, 1 *Pseudomonas aeruginosa*, 1 *Enterobacter complex* and 1 *Acinetobacter manniuii*) produced 93 different patterns by ERIC fingerprinting (Fig. 3 and 4).

Table 1. Sequence and concentration of forward and reverse primers

| Primers | Primers sequences | Product size | References |
|-------------|--------------------------------------|--------------|--|
| 16Sr RNA-F | 5' CCAGCAGCCGCGGTAATACG 3' | 300 bp | Jun <i>et al.</i> (2010) |
| 16Sr RNA-R | 5' TACCAGGGTATCTAATCC 3' | | |
| 16Sr DNA-F | 5-AGAGTTTGATCCTGGCTCAG -3' | | |
| 16Sr DNA- R | 5-ACGGCTACCTTGTTACGACTT-3' | 1500 bp | Behbahani <i>et al.</i> (2014) Rathinasamy <i>et al.</i> (2014) |
| ERIC1 | 5'-ATG TAA GCT CCT GGG GAT TCA C-3' | | |
| ERIC2 | 5'-AAG TAA GTG ACT GGG GTG AGC G- 3' | | |



Fig. 1. *A. hydrophila* and *V. cholerae* on Culture Media such as (A) *Aeromonas hydrophila*-HNK2 isolate LC194876 on Blood agar medium. (B) *V. cholerae* HNK3LC194877 isolate on Chromo agar

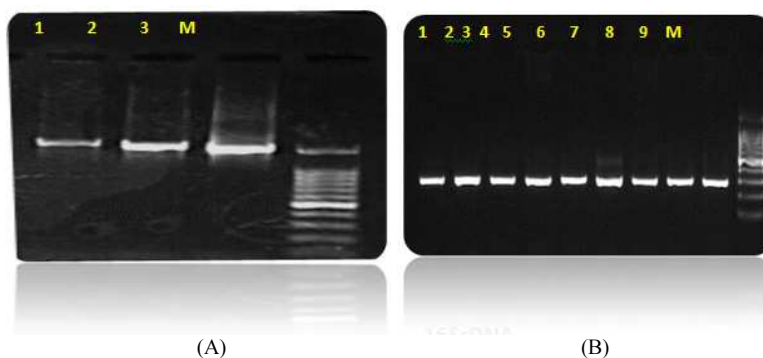


Fig. 2. Agarose Gel Electrophoresis (1.5%) of PCR Amplified of *16SrDNA* and *16S r RNA* Genes (1500bp, 300bp) respectively of *A. hydrophila* Isolates for (45) min at (100) volt A. *16SrDNA* gene (1500) bp. (M): 100-1500 bp Lane: 1, 2, 3 *A. hydrophila* B. *16SrRNA* gene (300) bp. (M): 100-1500 bp Lane (1,2,3,4,5,6,7,8,9) *A. hydrophila*

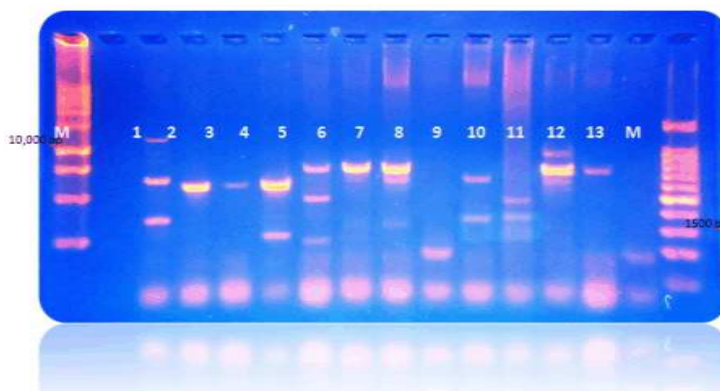


Fig. 3. Agarose Gel Electrophoresis (1.5%) of DNA Fingerprinting ERIC-PCR Genes Lane: (M) Marker 100-1500 bp and Marker 250-10.000 bp, Lane: (1,2,3,4,5,6,7,8,10,11,12) positive results of *A. hydrophila*, Lane: (13) *A. sobria* (9) *V. cholerae* isolates for (45) min at (100) volt

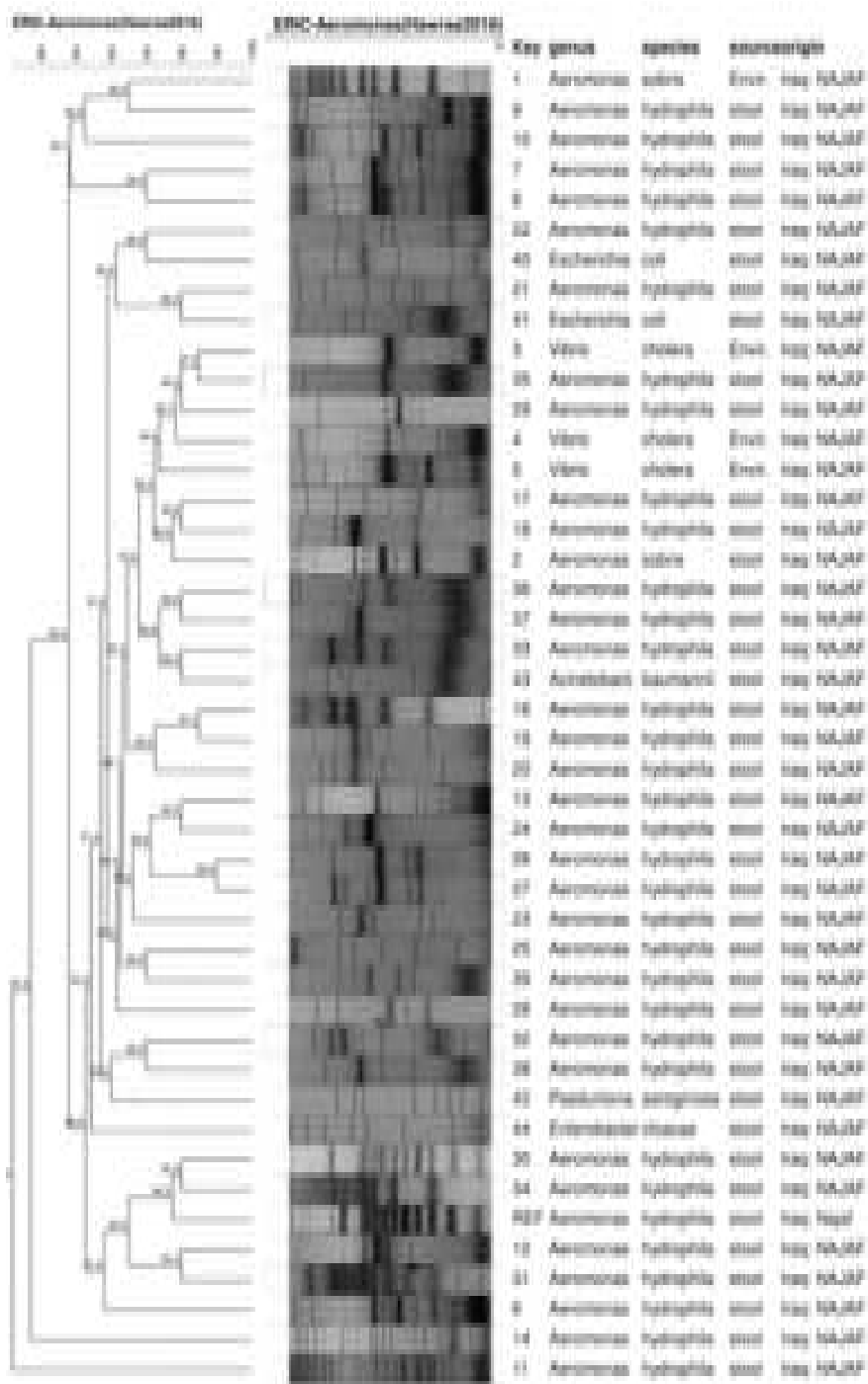


Fig. 4. Dendrogram UPGMA Phylogenetic representing Genetic Relationships between All (*Aeromonas* spp, *V. cholerae* and others isolates based on ERIC-DNA fingerprints

The fingerprinting patterns of the isolates were constructed using cluster analysis the UPGMA (group method) using average linkages Number of different bands (Similarity coefficient), 1% tolerance (Fig. 4). Dendrogram of phylogenetic analysis revealed the diversity of all isolates in the Najaf. The percentage level

of similarity clearly showed that the isolates examined by species were divided into (8) distinct cluster numbers, in addition to (4) single isolates, that clustered at a similarity level of (93%). Cluster I was the largest characterized by domination of phylogenetic group and specifically subgroup.

Sequencing and Analysis of 16SrDNA and 16SrRNA Gene Sequences

A. hydrophila, *A. sobria* and *V. cholerae* isolates were examined by sequencing technology to diagnosis of isolates and record it by (*16SrDNA*, *16SrRNA*) genes. All isolates were success in processing of a good running of sequencing by a Company DNA-Macrogen/Korea. The results were indicated to the first Iraqi isolates after compared in Gene Bank/BLAST is available at the NCBI online, using Nucleotide/Blast and recorded as the first sequencing in Gene-Bank/NCBI, DDBJ and ENA (INSDC).

16SrDNA and *16SrRNA* genes were successfully amplified using specific PCR primers for *A. hydrophila*, *A. sobria* and *V. cholerae* isolates which observed of results, which showed PCR amplification for *16SrDNA* and *16SrRNA* genes, which have a specific products (1500 and 300) bp respectively. Results analysis of the sequencing were revealed discovering new strains and recorded for each gene of isolates; *A. hydrophila*, *A. sobria* also *V. cholerae* isolates were recorded as the first sequencing in Gene-Bank by Accession numbers in Gene-Bank/NCBI, DDBJ and ENA (INSDC):

Accession numbers: Gene bank/NCBI/Nucleotide
Gene bank: LC194875 *Aeromonas sobria*-Hnk1
Gene bank: LC194876 *Aeromonas hydrophila*-Hnk2
Gene bank: LC194877 *Vibrio cholerae*-Hnk3
Gene bank/NCBI, DDBJ: ENA (INSDC) USA, these
Accession numbers as showed in index (1) and (2).

Discussion

Identification of *A. hydrophila*, *A. sobria* and *V. cholerae* depending on the morphology, biochemical tests, VITEK@2GN ID system and molecular identification. The colonies are green with black center *Aeromonas* isolates on Aeromonas media (Carriero *et al.*, 2016).

PCR technique has been amplify genes of *16SrDNA* and *16SrRNA* genes with genomic DNA of *A. hydrophila*, *A. sobria* and *V. cholerae* and other bacteria. The current results of all isolates diagnosis by PCR technique for detection both *16SrDNA* and *16SrRNA* clarify of *A. hydrophila*, *A. sobria* and *V. cholerae* and other bacteria, producers carrying *16SrDNA* and *16SrRNA* genes.

Singh *et al.* (2012) who noted that the ribosomal mainly *16SrRNA* gene has be a stable and specific for the identification of *A. hydrophila*, *A. sobria* bacteria. The present results are agree with (Al-Fatlawy and Al-Ammar, 2013; Boustanshenas *et al.*, 2016).

ERIC Sequence greater heterogeneity among the clinical and environmental isolates of *A. hydrophila*, *A. sobria* and *V. cholerae* and other have been demonstrated by ERIC DNA Fingerprinting. The isolates

revealed a clear structure on this basis, conclusion that the isolates of *Aeromonas* and other bacteria having genetically heterogeneous (Rathinasamy *et al.*, 2014).

Our results demonstrated that DNA Fingerprinting with ERIC primers amplification bands ranging in size (87 bp to 8000 kb). The 44 isolates produced 93 different patterns by ERIC fingerprinting (Fig. 4). The fingerprinting patterns of the isolates were constructed using cluster analysis the UPGMA (group method) using average linkages Number of different bands (Similarity coefficient), 1% tolerance.

Dendrogram of phylogenetic analysis revealed the diversity of all isolates in the Najaf. The percentage level of similarity clearly showed that the isolates examined by species were divided into (8) distinct cluster numbers, in addition to (4) single isolates, that clustered at a similarity level of (93%). Cluster I was the largest characterized by domination of phylogenetic group and specifically subgroup.

A. hydrophila, *A. sobria* and *V. cholerae* isolates were examined by sequencing technology to diagnosis of isolates and record it by (*16SrDNA*, *16SrRNA*) genes. All isolates were success in processing of a good running of sequencing by a Company DNA-Macrogen/Korea. The results were the first Iraqi isolates after compared in Gene Bank/BLAST is available in NCBI/Nucleotide/Blast and recorded as the first sequences in Gene-Bank/NCBI, DDBJ and ENA (INSDC).

16SrDNA, *16SrRNA* and *hlyA* genes were successfully amplified using specific PCR primers for *A. hydrophila*, *A. sobria* and *V. cholerae* isolates which observed of results which showed PCR amplification for *16SrDNA* and *16SrRNA* genes, which have a specific products (1500 and 300) bp respectively, as index (3).

Results analysis of the sequencing were revealed for each gene of isolates; *A. hydrophila*, *A. sobria* also *V. cholerae* isolates and recorded as the first Iraqi sequencing in Gene-Bank by Accession numbers in Gene-Bank/NCBI, DDBJ and ENA (INSDC).

All these sequences Recording of Iraqi sequences in NCBI-Gene-Bank and DDBJ of INSDC *A. hydrophila*, *A. sobria* and *V. cholerae* isolates sequences were isolated from clinical specimens and hospitals environment in NAJAF city and each sequence have Accession number (No. Gene bank: LC194875 *Aeromonas sobria*-Hnk1, LC194876 *Aeromonas hydrophila*-Hnk2 and LC194877 *Vibrio cholerae*-Hnk3) Gene bank/NCBI, DDBJ and ENA (INSDC) USA as showed in appendix (1), (2) and (3).

16SrDNA, *16SrRNA* genes sequence submitted to Gene bank. The results of these sequences were analyzed and examined by professional staff in Gene bank/NCBI, DDBJ and ENA (INSDC). All these sequences accepted in Gene bank and each sequence take accession number.

These results recorded and published in the International Nucleotide Sequence Database Collaboration (INSDC).

Local isolates in Najaf do not studying by sequences technology in past, therefore, the current study is discovering new isolates by contamination between patients and environments so that demonstrated these isolates in Najaf.

Conclusion

- The frequency of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates in Najaf were higher among clinical and environmental isolates
- Identification by VITEK@2GN card system and molecular technique is necessary for detection of pathogenic bacteria between clinical and environmental samples
- The ERIC-DNA Fingerprinting band pattern is an adequate tool for epidemiological investigations of *A. hydrophila*, *A. sobria* and *V. cholerae* isolates

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Author's Contributions

Hawraa Natiq Kabroot AL-Fatlawy: Research of this work, contribution in all work. Participated in all experiment, coordinated the data-analysis and contributed to the writing of the manuscript and designed.

Hawraa Abdalameer Aldahhan: Supervisor of this work.

Ali Hmood Alsaadi: Supervisor of this work in DNA laboratory.

Ethics Approval and Consent to Participate

Ph.D. student, Hawraa Natiq AL-Fatlawy Submitted the study was approved by University of Kufa and University of Babylon, the Faculty of Sciences and supported by Prof. Dr. Ali. Hmood,

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Appendix

Index 1. Recorded of Iraqi sequence of 16SrDNA gene of *A. sobria* HNK1 recorded in Gene-Bank/NCBI, DDBJ and ENA (INSDC)

LC194875.1
Nucleotide

GenBank

Aeromonas sobria gene for 16S ribosomal DNA, partial sequence, strain: HNK1
GenBank: LC194875.1

FASTA GenBank

LOCUS LC194875 1113 bp DNA linear NCT 02-DEC-2016
DEFINITION *Aeromonas sobria* gene for 16S ribosomal DNA, partial sequence.
STRAIN: HNK1.
ACCESSION LC194875
VERSION LC194875.1
KEYWORDS .
SOURCE *Aeromonas sobria*
ORGANISM *Aeromonas sobria*
Bacteria; Proteobacteria; Gammaproteobacteria; Aeromonadales;
Aeromonadaceae; Aeromonas.
REFERENCE 1
AUTHORS Al-Fatlawy, N.N., Al-Jabbar, H.A. and Alisawi, A.H.
TITLE Discovery New strains of *Aeromonas hydrophila* and *Vibrio cholera* in Iraq
JOURNAL unpublished
REFERENCE 2 (Chen & 1992)
AUTHORS Al-Fatlawy, N.N., Al-Jabbar, H.A. and Alisawi, A.H.
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ORCID: <https://orcid.org/0000-0001-9111-1111>
FEATURES
source 1..1113
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/mol_type="genomic DNA"
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