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Molecular Identification of *Aeromonas* spp. Collected from Taif Province of Saudi Arabia

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Abstract: ***The current study has been conducted for amplifying and sequencing approximately 750 nucleotides from 16S nuclear ribosomal DNA gene in order to identify the available bacterial strains inhabiting the water bodies in wades of Taif region in Saudi Arabia. Blast program was searched for defining to which bacterial strain the obtained data belong. The sequences from very closely related taxa were, therefore, aligned manually with the target sequence and subjected to bioinformatics manipulation in order to construct their genetic relationship. Two statistical methods (maximum-parsimony-MP and neighbor-joining-NJ) were used to execute the most likely relationship. One hundred forty one nucleotides varied among the examined strains under parsimony criteria of which 69 were parsimony-informative and 72 were parsimony-uninformative. A single NJ tree was obtained with slight difference in topology with that obtained from maximum parsimony. Both trees agreed in their topology regarding the clustering of the available bacterial strains. The NJ tree supported the clustering of the current strain to A. veronii with strong bootstrap probability. The present study therefore revealed the possibility that the current identified strain could be A. veronii or a new species. More molecular data and taxon sampling are necessary to identify the current bacterial strain with high resolution***

Keywords: *Aeromonas veronii*, 16S nuclear ribosomal DNA gene, water, Molecular identification

1. Introduction

The genus *Aeromonas* comprises ubiquistes bacteria considered indigenous to aquatic environnements [1]. Members of this genus have the ability to inhabit surface water (rivers, lakes), sewage, drinking water (tap and bottled mineral), thermal waters and sea water [2] [3] [4] . *Aeromonas* is also considered as pathogenic which can cause various types of diseases for human being [5] [1]. Among the leading pathogenic species are *A. hydrophila*, *Aeromonas caviae* and *A. veronii* [5]. *A. veronii* strains were isolated from clinical sources like secretions of dead bodies[6] . Seven species were isolated from the River Cocó in Brazil and were identified as *A. veronii sobria*, *A. veronii veronii*, *A. trota*, *A. media*, *A.sobria* and *A. hydrophila* [7. *Aeromonas* from drinking water réservoirs were also isolated and identified as *A. sobria* and *A. poppoffi* [8] [9]. *A. encheleia* and *A. allosaccharophila* and *A. hydrophila* were isolated from collective reservoirs and wells used as drinking water sources in a peri-urban area[10]. *A. veronii* is gram negative motile and rod shaped. Its strains can be found in various environment such as water sources and soil. *A. veronii* was isolated from patient's stool in southern Taiwan[11]. [12] have identified genes in *A. veronii* required for colonization of the medicinal leech. Recently, some virulence genes were screened for *A. veronii* [3]. Positive rate of virulence gene of A. veronii and *A. hydrophila* was compared between isolates from diseased fish and water environment.

The ribosomal 16S rRNA has been used as a specific molecular marker for different bacterial identification and it is useful for the bacterial molecular taxonomy[14] [15] [16] [17]. It is considered as a common taxonomical target due to its dual characters by having some conserved and some variable regions. The present study aimed to sequence this gene for some collected bacterial samples from water bodies in Taif region of Saudi Arabia in order to identify the available strains molecularly.

2. Materials and Methods

2.1 Isolation of the strain

In this research the current bacterial strain was isolated from different sources of drinking water from Taif region. The enrichment isolation technique was used to isolate this strain. The isolated colonies were picked up from plates according to their different form and purified by sub culturing onto fresh nutrient-metal agar plates using the streak-plate technique. Seventy isolates were screened from initial level of heavy metal supplemented LB medium (Nutrient agar). It consisted of 0.3 g beef extract (minerals and carbohydrates) , 0.5 g peptone (protein and nitrogen source), 0.5 g NaCl, 1.5 g agar and 100 ml distilled water. 1 ml from each sample was incubated for 48 hr at 30 ºC. Colonies were randomly picked from the plates, sub-cultured in nutrient agar slants and subjected for further characterization.

2.2 DNA extraction, PCR and sequencing

Genomic DNA was isolated and analyzed from bacterial isolates by the method of Chen and Kuo [11] ~~(1993)~~. Bacterial 16S rRNA gene was amplified by using the universal primers (F 5'-AGA GTT TGA TCC TGG CTC AG-3' and R 5'-GGT GTT TGA TTG TTA CGA CTT-3'). PCR was performed with a 50-µl reaction mixture containing 1-µl(10 ng) of DNA extract as a template, each primer at a concentration of 5 mM, 25 mM MgCl2 and dNTPs at a concentration of 2 mM, as well as 1.5 U of *Taq* polymerase and buffer used as recommended by the manufacturer. After the initial denaturation for 5 min at 94^{o}C, the samples were incorporated into 35 cycles consisting of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 5 min. PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis in 1x TAE buffer with ethidium bromide (0.5 µg/ml).

The PCR products were purified from gel with the use of Biospin gel extraction kit (BioFlux) according to the Kit Manual. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM. BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq-DNA polymerase (FS enzyme) (Applied Biosystems) following the protocols supplied by the manufacturer were used. A single-pass sequencing was performed on each template using the last mentioned PCR-primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

After reading the targeted gene, the nucleotide sequences have been treated with different software programs (DNASIS, MacClade and PAUP) that enabled to detect genetic relatedness between different strains. The sequenced genes were tested by BLAST program to check their relatedness to the published genes for other *Aeromonas* found in the Genbank database. The counterpart sequences of 16S rRNA gene from closely related bacterial strains were taken with thier accession numbers (NR_102789, NR_116586, NR_037013, NR_117303, NR_025945, NR_037014, NR_118043, NR_119042, NR_044846, NR_074924 and NR_074843) for the necessary alignments and tree construction. *Zobellella taiwanensis* (NR_043630) and *Ewingella americana* (NR_104925) were used as outgroups for tree rooting. The obtained DNA sequences were aligned separately and manually using MacClade v.4. The unalignable and gap-containing sites were deleted so that 748 bp were left for analyses. The tree analyses were done by Maximum-Parsimony (MP) and Neighbor- Joining (NJ) methods with PAUP* 4.0b10 [18] by heuristic searches with the TBR branch swapping and 10 random taxon additions, respectively. The bootstrapping replicates were set to be 5000 with simple additions for the Neighbor-Joining method.

3. Results and discussion

A total of 746 bp from 16S rRNA gene have been amplified and sequenced for the available bacterial strains in this study. The Blast program has been searched for identifying the present strain. This strain could be identified as *Aeromonas* where its identity to the congeneric taxa was found to be 99%. The same fragment for some taxa acquiring an identity of 99 to 95 % with the current strain were selected from Blast output for tree analyses. Two of the taxa acquired lower than 95% identity were used as an outgroup.

The collected sequences were manipulated and aligned by DNASIS and MacClade v.4 programs. The output file was analyzed by PAUP program [18] for constructing the genetic relationship and the obtained tree was described (Fig. 1). The sequenced fragment (746 bp) was deposited in NCBI GenBank database with an accession number (LC015098). The aligned data showed base frequencies of A = 18.7%, C = 33.9%, G = 22.2% and T = 25.2%. Of the 746 nucleotides, 605 were constant and 141 were variables. Seventy two of the variable sites were parsimony uninformative and 69 were informative under parsimony criterion. Based on the parsimony analysis, the strain showed sister relationship with almost all *Areomonas* species collected from the database but without strong statistical evidence (Fig. 1). However, a single neighbor-joining tree (Fig. 2) was obtained with reasonable statistical supports. The tree showed clustering of the present strain with *Aeromonas veronii* and the bootstrapping support was 94%. It is therefore evident to accept the close relationship between the current strain and *Aeromonas veronii*. The estimated genetic distance showed the lowest distance of 0.005 between the current strain and *A. veronii* (Table 1) indicating the concordance of the genetic distance and the tree topology.

The present study used the 16S rDNA gene since this gene has been used to identify and characterize several bacterial strains from a wide diversified environment [19] [20]. *Aeromonas* spp. were isolated from drinking water [17] [21] [22] isolated *A. veronii* strain PG01 from industrial wastes using the 16S rDNA sequence and have found it can be used to secrete extracellular protease. [15] has identified 67 environmental *Aeromonas* strians using the same gene. PCR-RFLP was used to identify and investigated the distribution of *Aeromonas* spp. in a trout farm [23] [24] have identifed the phynotypic and the genotypic framewark of *Aeromonas* spp. from the aquatic environment. [25] used the same gene to identify the antibotic resistant *Aeromonas*.. [26] also have identified the pathogenic *Areomonas* that is infecting the farm fish.

In conclusion, the niegbhor-joining tree and the pairwise distance that have been obtained from the 16S rDNA gene data supported that the current identifed strain could be *A. veronii* or it could be a separate species. More molecular data are necessary to identify, with more accuracy, the studied strain. The importance of studying the current strian has been raised since recent findings demonstrated increased antibiotic resistance among potentially pathogenic strains of aeromonads, illustrating an emerging potential health concern [27] . The behaviuor of the current strain has been observed that it is resistant to haeavy metals and antibiotics.

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Table 1: Pairwise genetic distances among different bacterial strains studied as calculated from the sequenced 16S rRNA gene

	A. veronii	*A. sp*	*A. fluvialis*	*A. diversa*	*A. schubertii*	*A. jandaei*	*A. salmonicida*	*A. tecta*	*A. allosaccharophila*	*A. enteropelogenes*
A. veronii	-	0.005	0.004	0.0054	0.011	0.008	0.02	0.015	0.009	0.024
A. sp		-	0.009	0.01	0.016	0.013	0.027	0.02	0.015	0.03
A. fluvialis			-	0.007	0.012	0.012	0.023	0.016	0.01	0.026
A. diversa				-	0.0054	0.011	0.027	0.02	0.015	0.028
A. chubertii					-	0.0149	0.015	0.02	0.02	0.032
A. jandaei						-	0.029	0.022	0.02	0.019
A.salmonicida							-	0.015	0.015	0.03
A. tecta								-	0.025	0.11
A.allosaccharophila									-	0.018

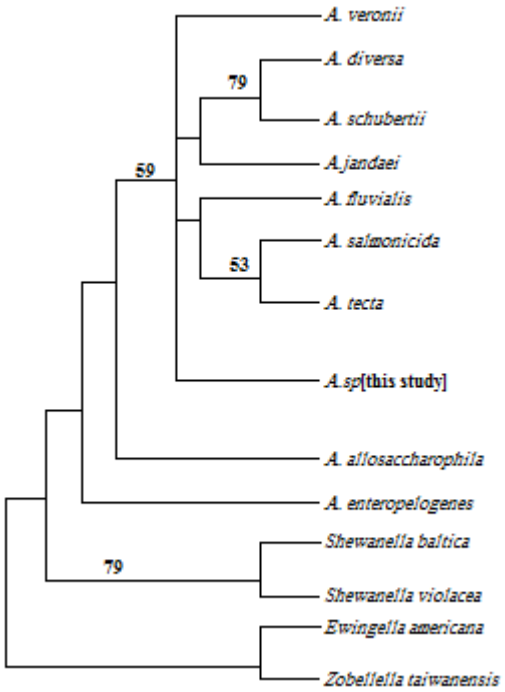

Figure 2: Maximum- parsimony tree constructed from 746 bp of 16S rRNA gene. Bootstrap probabilities are shown on branches when they are over 50%.

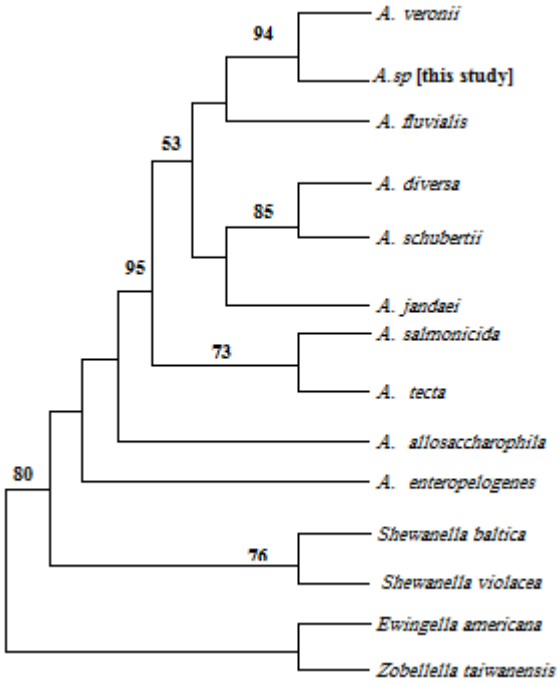

Figure 1: Neighbor- joining tree constructed from 746 bp of 16S rRNA gene. Bootstrap probabilities are shown on branches when they are over 50%.

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