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Study of microbial diversity in Mediterranean Sea water samples by the use of metagenomics technique

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Abstract
Microbes were the first organisms on Earth but the diversity of the microbial world is largely unknown, with less than one-half of 1% of the microbes have been estimated. The goal of this study was to examine the bacterial diversity in water samples by metagenomics technique which came out in recent years. Metagenomics is considered as a powerful method to study the bacterial diversity in environmental directly without culturing it. Four water samples of Mediterranean Sea were selected for comprehensive metagenomics exploration and nucleotide sequencing was performed on the microbes captured by sanger shut gun 16s rRNA gene and analysis for Phylogenetic approaches were also used to determine relationship between all types of bacterial sequence and provides unprecedented insight into bacterial diversity, which implausible to culture. The results show four new strains were pathogenic in nature. The results show four strains *Streptococcus anginosus*, *Vibrio vulnificus*, *Staphylococcus aureus* and *Vibrio vulnificus* were obtained in the study.

Keywords: metagenomics, mediterranean sea, 16s rRNA etc.

1. Introduction
Water represents a big part of the system on our planet earth. Water is one of the most important bacterial habitats on earth. Marine microbes have essentially contributed to global biomass, nutrient cycling and biodiversity since the early history of the Earth. The sea floor also offers many habitats for microorganisms. Considering this variety of habitats, the composition of marine microbial communities may be expected to vary in the different ecosystems. Yet, there has been so far very few information on the distribution of microbes in and across different ecosystems [8]. There is a growing interest in the role of marine microorganisms in biogeochemical processes, biotechnology, pollution and health. Due to the key role that bacteria play in the marine biogeochemical cycling and food-webs, advances in the study of the marine bacterial diversity provide fundamental information for a better understanding of the marine ecosystem functioning and the implementation of predictive models. The Mediterranean Sea is one of the most important environments for such microbes. As a part of this study, four water samples were collected to extract genomic DNA by metagenomics technique. The Mediterranean Sea is inhabited by very distinctive bacteria that tolerate environmental extremes and could have some potential industrial applications [1-3]. Metagenomics is emerging as a powerful method to study the function and physiology of the unexplored microbial biosphere, because more than 99% of total microbes are not known till now. In recent years the development of genomics has introduced new techniques and greater possibilities in the study of microbial diversity in the environment, and those techniques metagenome, Metagenomics is the name of new technology in the world of genetic engineering field that work on direct isolation of nucleic acids from different environmental samples. Aquatic environmental microbial diversity is a key component of water ecosystem and function [6, 13].

Metagenomics provides access to the functional gene installation of microbial communities and thus gives a much broader description than phylogenetic surveys, which are often based only on the diversity of one gene, for example the 16S rRNA gene. The term “Metagenomics” was first coined by Handelsman and his colleagues in their study of natural products from soil microbes [5].

2. Materials and Method

1. Date and place of collection of the water samples

Four surface water samples were collected from Mediterranean Sea at the city coast of Tripoli, Libya during November, 2015. The four water samples were designated as E, F, G and H.

2. Extraction of DNA by metagenomics technique from the water samples collected from Indian Ocean

The microbial DNA was extracted by metagenomics technique from the water sample obtained from Mediterranean Sea. The DNA was isolated directly without culturing the microbes into culture medium. Debris was removed from the collected water sample (100 ml) then it was poured through Micro cloth filtration material which is having size of 0.45µm per pore. This step was conducted to trap the microbes onto the filter membrane. 2µl of Tween 20 was added to 1 ml Distilled water. Here Tween 20 acts as filter wash buffer. Immediately, vortex the tube and transfer the cell suspension to a clean micro centrifuge tube. Resuspend the cell pellet in 300 µl of TE Buffer, than add 2µl of Ready-lyse lysozyme solution and add 1µl of RNase A to the cell suspension. Mix them by vortexing and incubate at 37 °C for 30 minutes. Now add 300µl of Meta-lysis solution (2X) and 1µl of Proteinase K to the tube. Mix them by vortexing and treat with 570µl of isopropanol. Now add 500µl of 70% ethanol to the pellet and re-suspend the pellet in 50µl of TE Buffer. Now centrifuge the sample at different time frames. Re-suspend the DNA pellet in 50 µl of TE Buffer and store for further work.

3. Agarose gel electrophoresis

Dissolve agarose powder in 1X TAE buffer. Wipe the gel casting tray & comb with ethanol. Pour the agarose solution in the gel casting tray and leave to solidify for 15-20 minutes. Mix the DNA sample (pellet) in 50µl of TE buffer. Load the samples in the wells carefully using pipettes in order that the gel should not be broken. Run the sample for 20-30 minutes. Remove the gel from the electrophoresis tank and observe the bands on UV transilluminator.

4. Polymerase chain reaction

Amplification of DNA was be performed in a total volume of 30µl along with 16s rRNA.

5. Sequencing analysis by bioinformatics tools

Sequencing analysis was performed by using NCBI BLAST tool and CLUSTAL omega tool. To construct the phylogenetic tree CLUSTAL OMEGA was used. Homepage of CLUSTAL OMEGA was opened from the URL www.ebi.ac.uk. Selected all the aligned sequences and click on the distance tree of result. A phylogenetic tree will appear along with your query sequence. Click on the name of any species, then on layout and finally on radial tree to get the structure of the phylogenetic tree.

3. Results and discussions

The result shows that good amount of genomic DNA was extracted from the samples obtained from Indian Ocean and similar results were recorded by [4]. The results obtained from after visualizing the bands in the gel electrophoresis have shown genomics DNA of sample E, F, G and H **(Figure 1).** It also shows that genomic DNA of more than 10kb, 8kb, 6kb, 1kb.

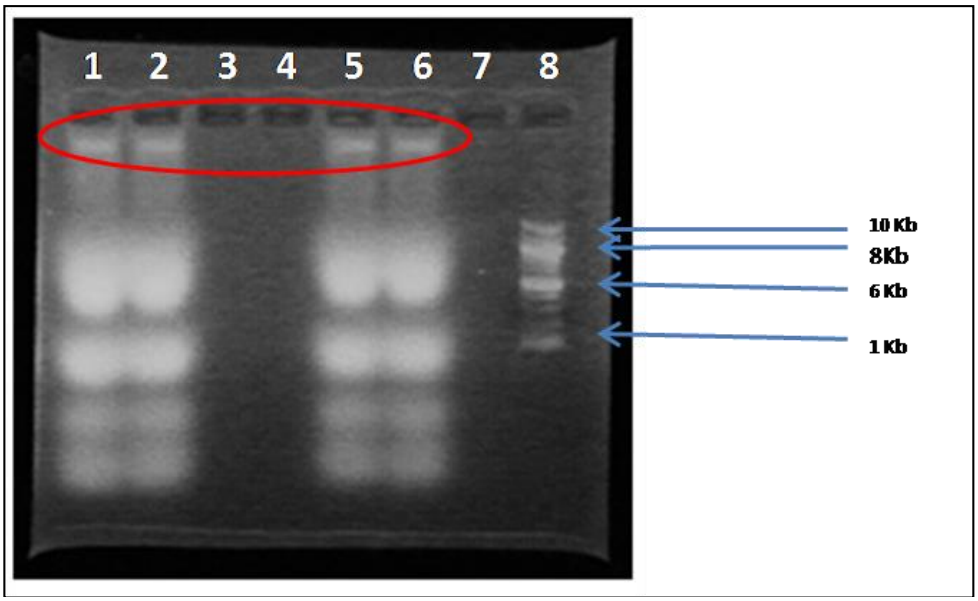

Fig 1: Results of the agarose gel electrophoresis for genomic DNA extracted from the Mediterranean Sea water samples designated as E, F, G and H.

Lane 8: 1kb DNA Ladder

Lane 1: Sample E (Genomic DNA) Size: More than 10 kb
Lane 2: Sample F (Genomic DNA) Size: More than 10 kb
Lane 5: Sample G (Genomic DNA) Size: More than 10 kb
Lane 6: Sample H (Genomic DNA) Size: More than 10 kb

2. Quantification of genomic DNA extracted from different water samples of Mediterranean Sea:

Quantification step was performed by using UV-Vis double beam spectrophotometer of wave length between 254-260nm and the significant DNA concentration in sample (Table 1).

Table 1: Result of the DNA concentration in water samples of Indian Ocean

S. No.	Sample ID	Variety of samples	Ratio	DNA concentration	Protein concentration
1	E	Mediterranean Sea	1.35	1.31	1.68
2	F	Mediterranean Sea	1.18	12.02	20.67
3	G	Mediterranean Sea	2.05	1.50	0.43
4	H	Mediterranean Sea	1.55	1.04	0.94

After the amplification, the genomic DNA was obtained from the four water samples shows the bands at 10kb, 8kb, 6kb and 2kb (Figure 2) with DNA ladder of 10kb.

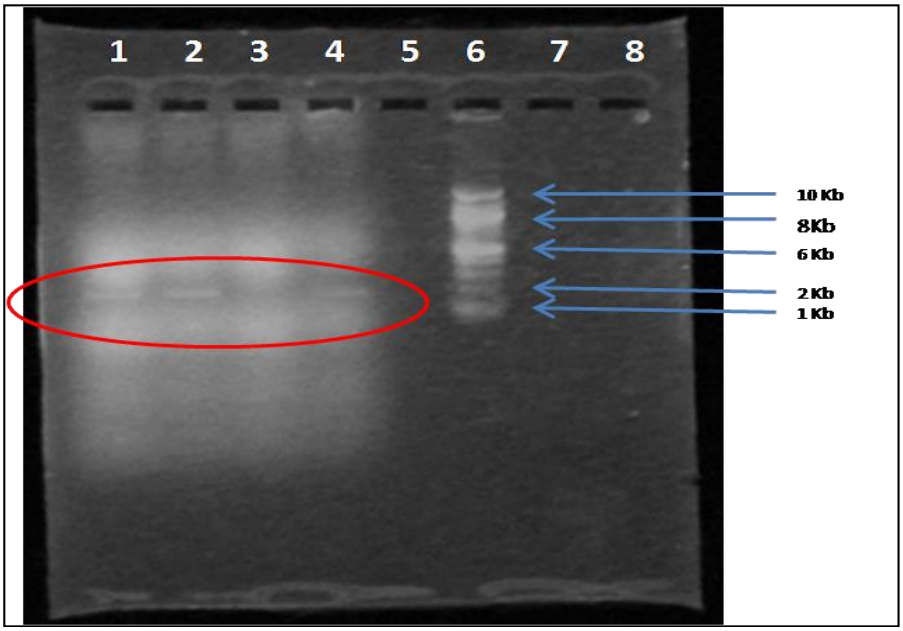

Fig 2: Agarose gel electrophoresis of amplified DNA products by PCR for the water samples namely E, F, G and H.
Lane 6: 1kb DNA Ladder

Lane 1: Sample E (PCR product) Size: 1.2-1.3 kb
Lane 2: Sample F (PCR product) Size: 1.2-1.3 kb
Lane 3: Sample G (PCR product) Size: 1.3-1.4 kb
Lane 4: Sample H (PCR product) Size: 1.3-1.4 kb

3. Sequencing of the amplified DNA products of the different water samples

The sequencing was performed by Sanger's method and the sequences showed similarity. The code sequences obtained are as follows (Table 2).

Table 2: Results of the sequences of the different water samples

S. No.	Sample ID	Samples description	Code sequence
1	E	Mediterranean sea	CG20160324E
2	F	Mediterranean sea	CG20160324F
3	G	Mediterranean sea	CG20160324G
4	H	Mediterranean sea	CG20160324H

4. Sequencing analysis by bioinformatics tools

(a) Result of the BLAST for the first sequence CG20160324E of the first water sample i.e. E

The sequence which was obtained from the sample E showed 98% similarity with *Streptococcus anginosus* 16s RNA partial sequence. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Streptococcus anginosus* 16s RNA partial sequence and relation with other bacteria (Figure 3).

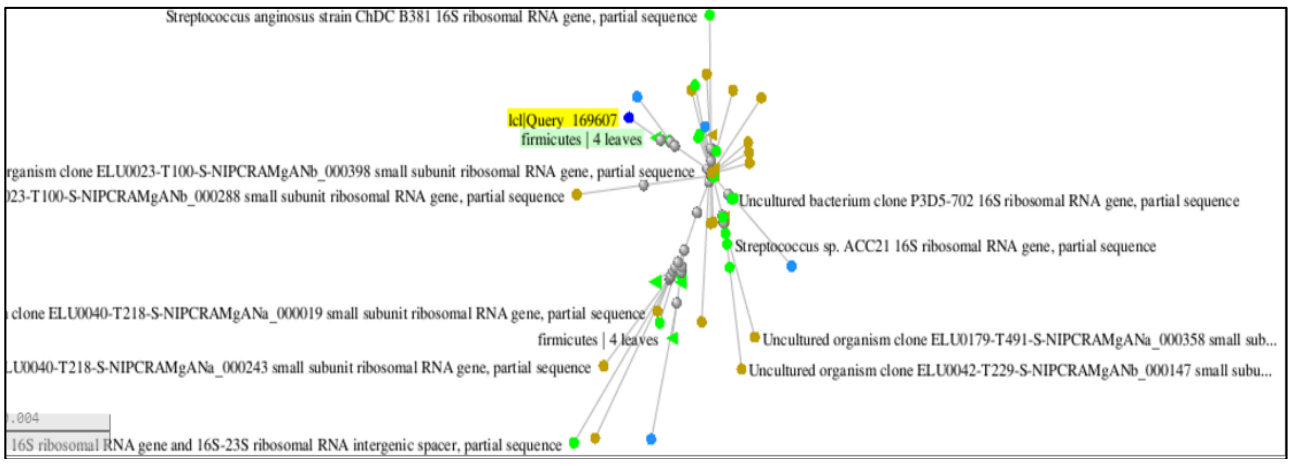

Fig 3: Phylogenetic tree of *Streptococcus anginosus* 16s rRNA partial sequence

After the analysis with the phylogenetic tree the observed result showed similarity to *Streptococcus anginosus.* Members of the group are gram-positive, catalase-negative cocci (like other members of the genus Streptococcus). They are non-motile facultative anaerobes that demonstrate variable hemolysis patterns (alpha, beta, or gamma) on sheep blood agar. Colonies are typically small (colony size less than 0.5 mm) Many strains demonstrate enhanced growth in the presence of CO_2, whereas some strains may require anaerobic conditions [9].

(b) Result of the BLAST for the second sequence CG20160324F of the second water sample i.e. F:

The sequence which was obtained from the sample F showed 97% similarity with *Vibrio vulnificus* strain ATCC 27562. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Pseudomonas meridian* gene 16srRNA and relation with other bacteria (Figure 4).

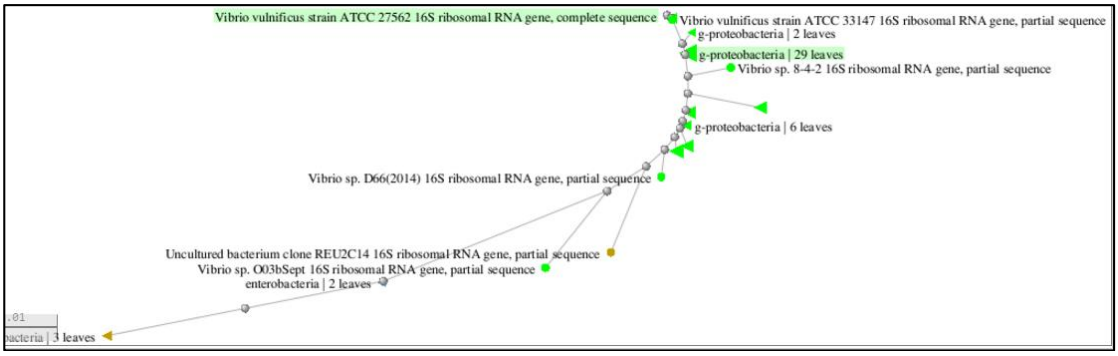

Fig 4: Phylogenetic tree of *Vibrio vulnificus* strain ATCC 27562 16s rRNA complete sequence

After the analysis of the second sequence with the phylogenetic tree the observed result showed similarity to partial sequence of result with *Vibrio vulnificus* strain ATCC 27562. *Vibrio vulnificus* is a gram-negative bacillus that only affects humans and other primates. It is in the same family as bacteria that cause cholera. *Vibrio vulnificus* is an etiologic agent in severe human infection acquired through wounds or contaminated seafood. The strains belonging to this species are divided into three biotypes according to their different biochemical and biological properties. *Vibrio vulnificus* is usually found in warm, shallow, coastal salt water in temperate climates throughout most of the world. This organism can survive in seawater and can produce wound infections [10].

(c) Result of the BLAST for the third sequence CG20160324G of the third water sample i.e. G:

The sequence which was obtained from the sample G showed 98% similarity with *Staphylococcus aureus subsp. aureus* strain FORC.001 complete genome. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these associations. The design of phylogenetic tree with *Staphylococcus aureus subsp. aureus* strain FORC.001and relation with other bacteria (Figure 5).

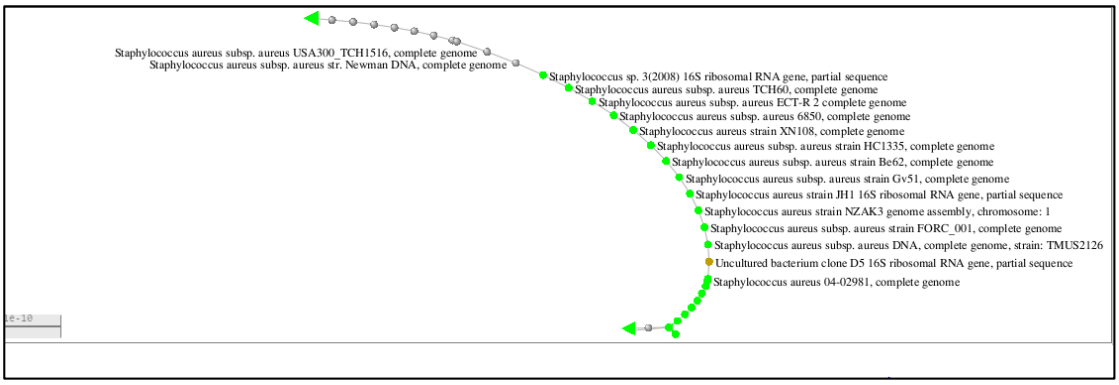

Fig 5: Phylogenetic tree of *Staphylococcus aureus subsp. aureus* strain FORC.001 complete genome

After the analysis of the third sequence with the phylogenetic tree the observed result showed similarity to partial sequence of result with *Staphylococcus aureus subsp. aureus* strain FORC.001 complete genome. It is gram-positive spherical bacteria that occur in microscopic clusters resembling to grapes like structure. *Staphylococcus aureus* is considered as the third most important cause of food-borne disorders in the world causing up to 9000 deaths. *Staphylococcus aureus* transmitted mainly through foodstuffs and the important cause of food contamination including milk products and beef. *Staphylococcus aureus* mainly invades through the nasal passages, but it is also found regularly in most other anatomical locales, including the skin, oral cavity and gastrointestinal tract. *Staphylococcus aureus* has developed resistance to most classes of antimicrobial agents. Penicillin is the drug of choice to treat *Staphylococcus* infections [11, 12].

(d) Result of the BLAST for the fourth sequence CG20160324H of the fourth water sample i.e. H

The sequence which was obtained from the sample H showed 98% similarity with *Vibrio vulnificus* strain ATCC27562 16s rRNA gene complete genome. To understand evolutionary relationship is a fundamental aspect of modern biology, with the phylogenetic tree being a primary tool for describing these

associations. The design of phylogenetic tree with *Vibrio vulnificus* strain ATCC27562 16s rRNA gene complete genome and relation with other bacteria (Figure 6).

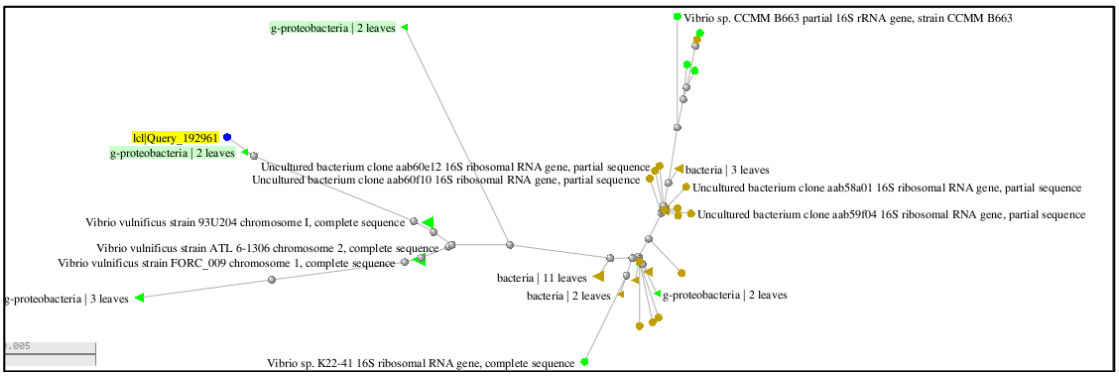

Fig 6: Phylogenetic tree of *Vibrio vulnificus* strain ATCC27562 16s rRNA gene complete genome

After the analysis of the fourth sequence with the phylogenetic tree he observed result showed similarity to partial sequence of result with the topology of the phylogenetic tree confirmed that they were members of the *Vibrio vulnificus* group. *Vibrio vulnificus* is a gram-negative halophilic bacterium natural inhabitant of estuarine and coastal waters. In healthy individuals, this pathogen may cause gastroenteritis or severe wound infections, leading to necrotizing cellulitis. *Vibrio vulnificus* infection often leads to septicemia, especially in immunocompromised individuals, case-fatality rates are greater than 50% for primary septicemia and about 15% for wound infections. *Vibrio vulnificus* has been recovered from fish shellfish, water and sediments of a widely range of temperatures and salinities [7].

5. Nucleotide sequence accession numbers

All nucleotide sequence data from Mediterranean Sea of this study were submitted to the DNA data bank of Japan (DDBJ) for getting the accession numbers of the four water samples.

Table 3: Results of the accession numbers of various water samples

Sample No.	Sample ID	Sample description	Code Sequence	Accession Number
1	E	Mediterranean sea	CG20160324E	LC140947
2	F	Mediterranean sea	CG20160324F	LC140948
3	G	Mediterranean sea	CG20160324G	LC140949
4	H	Mediterranean sea	CG20160324H	LC140950

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