Identification of Bacterial Species in Kuwaiti Waters Through DNA Sequencing

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Abstract: With an objective of identifying the bacterial diversity associated with ecosystem of various Kuwaiti Seas, bacteria were cultured and isolated from 3 water samples. Due to the difficulties for cultured and isolated fecal coliforms on the selective agar plates, bacterial isolates from marine agar plates were selected for molecular identification. 16S rRNA genes were successfully amplified from the genome of the selected isolates using Universal Eubacterial 16S rRNA primers. The resulted amplification products were subjected to automated DNA sequencing. Partial 16S rDNA sequences obtained were compared directly with sequences in the NCBI database using BLAST as well as with the sequences available with Ribosomal Database Project (RDP).

1. Introduction

Bacteria are key microbes in marine microbiology that play fundamental roles in the ocean environment. Apart form being the base of the food chain, supplying more than half of the world's oxygen, controlling the role of marine nutrients, and being major processors of greenhouses gases, they are also rapidly responsive to changes in their ecosystem, making bacteria an ideal indictor of marine change [1][2]. One such case is the microbialization of bacteria in contaminated waters over the past decade. The growing pathogenicity of marine microbes can cause disease in both marine and human life. Upon contact or consumption of specific bacteria, humans can experience cramps, nausea, diarrhea, tuberculosis, legionella pneumonia, typhoid, and cholera [3]. Bacterial diseases of fish include-but are not limited to: hemorrhagic septicemia, vibriosis, and columnaris [4]. The susceptibility of infection from pathogenic bacteria reinforces the importance of water purification before industrial, agricultural, or domestic applications. It is also ideal that water treatment processes use purification methods that limit leftover waste, as brine sediments often contain pathogens not killed off during treatment, and disposing them back into the ocean reintroduces the harmful bacteria into the aquatic environment in abundance.

There is growing interest in marine bacteria in the scientific world because pathogenic, or otherwise, their and identification serves many purposes. Firstly, extremophiles can be used as a measure of water pollution levels or aquatic condition because these species only grow in extreme environments. For example, halophilic bacteria only thrive in waters of high salt concentration, and are an indicator of overly high salinity. Next, being aware of pathogenic bacteria and locations were specific genera reside allows researchers, scientists and doctors to better prevent disease that could be contracted in the area-some of which can be very dangerous. Treatment plants can also better allocate purification methods for more effect water treatment with an understanding of the bacterial genera residing in specific marine regions. For example, bacteria killing methods of purification-such as

electrochemical remediation, will be more efficient in treating water samples containing pathogens than other methods that do not eliminate bacteria. Employing target specific ways of water purification is more effective, efficient, and economical. Finally, bacterial identification is also important because many bacterial species such as: Marinobacter hydrocarbonoclasticus, Carboxydothermus hydrogenoformans, and Sphingomonas harbor properties that can be industrially useful in pharmaceutical, biotechnological, agricultural, chemical, and environmental fields.

The purpose of this study is to investigate the diversity and properties of marine bacteria species from Salwa Beach (N1), North West (N2) and Sulaibikhat Beach (N3) by directly culturing seawater samples, PCR amplification of 16S rRNA gene, and DNA sequencing.

2. Methodology

2.1. DNA Growth and Preparation

Bacteria from each beach was first grown by spreading 3µL of each sample onto separate marine agar plates, then incubated at 28°C in an bacteriological incubator for 24 hours. Grown bacteria cultures were then collected and mixed into cell pellets of 10µL of sterile distilled water. 48 samples of bacteria were collected from each beach.

Cultivation was also attempted on MacConkey, and nutritional agar plates. However, results indicated that bacteria could not be cultured on said plates, which made the identification of fecal coliforms based on culturing conditions and microscopical morphologies impossible.

2.2. PCR

Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the following universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 5' ACGGCTACCT TGTTACGACTT 3'. An ABI PCR System 9700 was used to perform the PCR protocol in a volume of 24 µL of bacteria strain identification solution mix containing 10 x 2.5µL buffer, 1.25µL magnesium chloride, 1µL dNTP, 0.05µL primer 1, 0.05µL primer 2, 0.1µL enzyme taq, and 19.05µL of distilled water per 1µL of template bacteria. Thermal cycling consisted of a 10 minute initial denaturation at 95°C, 30 cycles of denaturation at 94°C for 30 seconds, 30 seconds of annealing at 60°C, and extension for 2 minutes at 72°C with a 10 minute final maintenance at 4°C [5].

2.3. Agarose Gel Electrophoresis

From each PCR product, an aliquot of 15µL was subject to 1.0% agarose gel electrophoresis containing 0.5 x TBE buffer (pH 8.0), and 5µL of ethidium bromide was used to stain the gel. Electrophoresis was conducted at 100 Volt, 400mA for 20-30 minutes with 0.8µL of 100bp DNA marker. After, the DNA bands were observed under ultraviolet light using gel documentation system [5].

2.4. Sequencing of 16S rRNA Genes

Sequencing reaction was carried out using ABI PRISM 310 Genetic Analyzer (PE applied Biosystems). For the sequencing reaction, Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was employed.

2.5. Data Analysis

Sequence analysis was performed with sequences in the NCBI database using BLAST as well as with sequences available with Ribosomal Database Project (RDP)

3. Results and Discussion

3.1. Salwa Beach

Total bacteria counts: 85

Table.1. Identified bacteria isolates in Salwa Beach after data analysis.

Isolate	Identified Bacterial Strain
N1-001	Uncultured bacterium clone ncd981d10c1
N1-002	Exiguobacterium acetylicum
N1-003	Cronobacter sakazakii
N1-004	Uncultured bacterium isolate KWSUB08_76 16S
N1-005	Marinobacter sp. MMRF630 16S
N1-006	Alteromonas macleodii
N1-007	Marinobacter litoralis
N1-009	Marinobacter sp. HOT2G5 16S
N1-010	Alteromonas sp. NBF17 16S
N1-011	Alteromonas macleodii
N1-012	Bacterium SCSIO13055 16S
N1-013	Bacterium SCSIO13055 16S
N1-014	Pseudoalteromonas sp. Bac192 16S
N1-015	Alteromonas macleodii
N1-016	Alteromonas sp. SS12.5 16S
N1-017	Phaeobacter caeruleus
N1-018	Enterobacter cloacae
N1-019	Alteromonas macleodii strain CSB14KR 16S
N1-020	Sphingomonas
N1-021	Spirometra erinaceieuropaei
N1-022	Alteromonas sp. OCN004 16S
N1-023	Hydatigera taeniaeformis
N1-024	Gamma proteobacterium
N1-025	Schistosoma rodhaini
N1-026	Erythrobacter citreus
N1-027	Exophiala mesophila 3-isopropylmalate dehydrogenase
N1-029	Tropicibacter sp. MCCC1A07686 16S
N1-030	Xanthomonas oryzae pv. Oryzae
N1-031	Pseudoalteromonas sp. F497 16S
N1-032	Pseudomonas sp. X3-2
N1-033	Pseudomonas psychrophila
N1-034	Colacium sp. Ungok033107C
N1-035	Citromicrobium bathyomarinum
N1-036	Candida catenulate
N1-038	Diphyllobothrium latum
N1-041	Schistosoma rodhaini
N1-045	Spirometra erinaceieuropaei
N1-046	Tetrapisispora phaffii
N1-047	Plasmodium falciparum

Table.2. Pathogenic bacteria found in Salwa Beach isolates after data analysis.

Isolate	Description
N1-003	Cronobacter sakazakii: causes cronobacter disease with fatality rates of 50-80%. Infants and immunocompromised adults are most susceptible [6].
N1-018	Enterobacter cloacae: Enterobacter cloacae infection has the highest mortality rate amongst all Enterobacter infections. It causes bacteremia, skin and soft-tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, CNS infections, ophthalmic infections, and lowers respiratory tract

	infections [7].
N1-021	Spirometra erinaceieuropaei: is a tapeworm that causes a parasitic infection called sparganosis [8].
N1-023	Hydatigera taeniaeformis: a parasitic tapeworm with cats as their primary definitive hosts [9].
N1-025	Schistosoma rodhaini: commonly known as blood-flukes, they are parasitic flatworms that cause schistosomiasis in humans-an infection considered by the World Health Organization to be the second most socioeconomically devastating parasitic disease infecting hundreds of millions worldwide [10].
N1-030	Xanthomonas oryzae pv. Oryzae: causes bacterial blight, a serious disease for rice, grass, and sedges worldwide [11].
N1-038	Diphyllobothrium latum: is the largest human tapeworm and the principle species of Diphyllobothrium causing diphyllobothriosis It infects humans through the somsuptiono of raw or undercooked seafood, but can also affect fish and other mammals [12].
N1-041	Schistosoma rodhaini: refer to N1-025
N1-045	Spirometra erinaceieuropaei: is a tapeworm that infects humans and domestic animals. In humans, is causes sparganosis [8].
N1-047	Plasmodium falciparum: is a protozoan parasite that is the deadliest of five human malaria species, being responsible for more than 85 % of all malaria cases, and has the highest mortality rate amongst all malaria species [13].

It can be inferred that Salwa beach is home to an abundance of tapeworms, as 6 out of 8 pathogenic bacteria identified were of such. Tapeworms, which generally reside in areas of inadequate sanitation is an indication of the area's contamination levels. Salwa beach is also a harbor. Many tapeworms could dwell inside the intestines of fish, so extra persuasions should be taken when eating undercooked seafood to prevent infections.

Table.3. Bacteria with industrial uses found in Salwa Beach isolates after data analysis.

Isolate	Description
N1-020	Sphingomonas: typically found in environments polluted with toxic compounds, they have the ability to utilize the contaminants as nutrients. Their biodegradative and biosynthetic capabilities have been utilized for numerous biotechnological applications including bioremediation of environmental contaminants for the production of extracellular polymers such as sphingans [7]. It is also effective in degrading degrade alkylated polyaromatic hydrocarbon (2-methylphenanthrene), commonly known as polystyrene plastic [14].
N1-031	Pseudoalteromonas sp. F497: capable of synthesizing biologically active molecules that produce compounds beneficial to eukaryotic organisms [15].
N1-046	Tetrapisispora phaffii: killer yeast that secretes a glycoprotein known as Kpkt that has antimicrobial activity with spoilage yeasts under winemaking conditions. It can be used to combat contaminating wild yeasts in food, to control pathogenic fungi in plants, and to develop novel antimycotics for the treatment of human and animal fungal infections in the medical field [16].

There is no pattern to the useful bacterial species identified in Salwa beach, but the particularly useful Sphingomonas was cultured and identified. Its unique ability to synthesize and degrade toxic compounds in water could be applied as an effective organism in water purification-a function that can be utilized in Kuwait's many water treatment plants.

3.2. North West Beach

Total bacteria counts: 112

Table.4. Identified bacteria isolates in North West Beach after data analysis.

Isolate	Identified Bacterial Strain
N2-001	Muricauda sp. SA1 16S
N2-002	Alteromonas macleodii strain G7C_40m_M_02 16S
N2-003	Myxococcus fulvus
N2-004	Marinobacterium stanieri
N2-005	Idiomarina aquimaris
N2-006	Marinobacter hydrocarbonoclasticus
N2-007	Spongiibacter marinus
N2-010	Flavobacteriales bacterium
N2-011	Marinobacter sp. NNA5 16S
N2-012	Alteromonas macleodii
N2-013	Corynebacterium humireducens
N2-014	Rhodobacteraceae bacterium
N2-015	Bacillus pumilus
N2-016	Schistosoma mattheei
N2-017	Muricauda sp. mur1 16S
N2-018	Flavobacteriales bacterium
N2-019	Marinobacter hydrocarbonoclasticus
N2-020	Marinobacter flavimaris
N2-021	Rhodobacteraceae bacterium
N2-022	Marinobacter Pelagius
N2-023	Rhodobacteraceae bacterium
N2-024	Loxodonta africana ALG12, alpha-1,6-mannosyltransferase
N2-025	Pectobacterium sp. SCC3193
N2-026	Brassica napus
N2-027	Schistosoma curassoni
N2-028	Marinobacter flavimaris
N2-029	Erythrobacter citreus
N2-030	Marinobacter hydrocarbonoclasticus
N2-036	Novosphingobium sp. SG8916S
N2-037	Marinobacter sp. G5-4a
N2-038	Rhodobacteraceae bacterium
N2-039	Mus musculus
N2-040	Solitalea canadensis
N2-041	Pseudoalteromonas sp. ECSMB85 16S
N2-042	Marinobacter flavimaris
N2-043	Spongiibacter marinus
N2-044	Schistosoma curassoni
N2-045	Cyanobium sp. NIES-981
N2-046	Marinobacter sp. DY57-1 16S
N2-047	Uncultured organism clone ctg_CGOF252 16S
N2-048	Roseovarius pacificus

Table.5. Pathogenic bacteria found in North West Beach isolates after data analysis.

Isolate	Description
N2-016	Schistosoma mattheei: a parasite of sheep, goats, and cattle in regions of West Africa [17].
N2-027	Schistosoma mattheei: refer to N2-016
N2-044	Schistosoma mattheei: refer to N2-016

Schistosoma mattheei was the singular pathogenic bacteria culture and identified in North West Beach, and it has no direct threat to either humans or marine life. The area of the collected sample

should be taken into consideration, as it is the closest to a direct polluting source (industrial power plant) with notably less fish than in popular fishing regions such a Salwa Beach. This could possibly explain the sample's lack of pathogenic bacteria, as many species of pathogens reside in marine life.

Table.6. Bacteria with industrial uses found in North West Beach isolates after data analysis.

Isolate	Description
N2-006	Marinobacter hydrocarbonoclasticus: are important to bioremediation because they can effectively degrade hydrocarbons-especially those found in alkanes and major components of oil as they are tolerant of high salinity and can grow aerobically and anaerobically [18]. The marinobacter hydrocarbonoclasticus' distinctive ability makes it ideal for treating recalcitrant pollutants [19].
N2-015	Bacillus pumilus: is used as an active ingredient in agricultural fungicides because its growth on plant roots prevents rhizoctonia and fusarium spores from germinating [20]. Bacillus pumilus proteases are also used in chemical, detergent, food, and leather industries as antimicrobials and antifungals.
N2-019	Marinobacter hydrocarbonoclasticus: refer to N2-006
N2-029	Erythrobacter citreus: is a potential candidate for removing poisons because it can resist and reduce the toxic compound tellurite [21].
N2-030	Marinobacter hydrocarbonoclasticuss: refer to N2-006

3 isolates of Marinobacter hydrocarbonoclasticus was found out of the 48 bacteria samples taken, suggesting that the species is abundant in North West Beach waters. Its properties are very useful in purifying Kuwaiti water, as it is able to degrade hydrocarbons found in oil. Oil is an abundant resource in Kuwait that frequently pollutes the county's water sources, but is difficult to remove once contaminated. Therefore, the potential of using bacteria to purify this recalcitrant pollutant is exciting and could perhaps be more cost efficient than currently employed methods.

3.3. Sulaibikhat Beach

Total bacteria counts: 126

Table.7. Identified bacteria isolates in Sulaibikhat Beach after data analysis.

Isolate	Identified Bacterial Strain
N3-001	Carboxydothermus hydrogenoformans
N3-002	Lactococcus garvieae
N3-003	Vibrio alginolyticus
N3-004	Vibrio parahaemolyticus
N3-005	Vibrio sp. H-188 16S
N3-006	Virgibacillus sp. SK33 16S
N3-007	Vibrio alginolyticus
N3-008	Vibrio alginolyticus
N3-010	Exiguobacterium undae
N3-012	Uncultured gamma proteobacterium
N3-013	Exiguobacterium undae
N3-014	Exiguobacterium undae
N3-015	Exiguobacterium undae
N3-017	Vibrio sp. 3677
N3-018	Vibrio alginolyticus
N3-019	Vibrio sp. B2-15-2 16S
N3-020	Vibrio sp. H-219 16S
N3-021	Vibrio sp. 3683
N3-022	Vibrio orientalis
N3-023	Vibrio natriegens

N3-024	Providencia rettgeri
N3-025	Clupea harengus translocase
N3-026	Anas platyrhynchos
N3-027	Drosophila takahashii
N3-028	Vibrio sp. sw9 16S
N3-029	Uncultured bacterium clone I3Q1XXJ01APUOI 16S
N3-030	Nippostrongylus brasiliensis
N3-031	Vibrio sp. 3677
N3-033	Vibrio alginolyticus
N3-034	Vibrio sp. B2-15-2 16S
N3-035	Vibrio sp. H-219 16S
N3-036	Vibrio sp. 3683
N3-038	Vibrio orientalis
N3-039	Vibrio natriegens
N3-040	Providencia rettgeri
N3-041	Clupea harengus
N3-042	Anas platyrhynchos
N3-043	Drosophila takahashii
N3-044	Vibrio sp. sw9 16S
N3-045	Uncultured bacterium clone I3Q1XXJ01APUOI 16S
N3-046	Nippostrongylus brasiliensis

Table.8. Pathogenic bacteria found in Sulaibikhat Beach isolates after data analysis.

Isolate	Description
N3-002	Lactococcus garvieae: is a zoonotic pathogen that causes infective endocarditis, spondylitis, osteomyelitis, and liver abscesses in humans. But mostly affects the Japanese yellowtail, rainbow trout, and grey mullet [22].
N3-003	Vibrio alginolyticus: like most Vibrio infections, this is a foodborne disease that causes vibriosis, otitis and wound infection [23].
N3-004	Vibrio parahaemolyticus: also cause vibriosis, along with gastrointestinal illness when ingested [24].
N3-007	Vibrio alginolyticus: refer to N3-003
N3-008	Vibrio alginolyticus: refer to N3-003
N3-018	Vibrio alginolyticus: refer to N3-003
N3-030	Nippostrongylus brasiliensis: is a gastrointestinal roundworm that primarily infects rats [25].
N3-033	Vibrio alginolyticus: refer to N3-003
N3-040	Providencia rettgeri: causes infections involving the urinary tract with symptoms of polyuria, hematuria, fevers, and dysuria. It has also recently been implicated as etiologic agents in traveler's diarrhea [26].
N3-046	Nippostrongylus brasiliensis: refer to N3-030

Species of bacteria from the Vibrio genus was especially prevalent in Sulaibikhat Beach, with 6 out of 10 identified isolates fitting into the category. Both Vibrio alginolyticus and Vibrio parahaemolyticus, along with Providencia rettgeri, are all foodborne infections. Therefore, precautions should be taken against eating undercooked food fished from this area.

Table.9. Bacteria with industrial uses found in Sulaibikhat Beach isolates after data analysis.

Isolate	Description
N3-001	Carboxydothermus hydrogenoformans: is an extremely thermophilic bacteria that feeds almost entirely on toxic carbon monoxide and water, and produces hydrogen gas as part of its metabolism [27]. Its ability to rapidly and efficiently conduct this conversion

	makes it highly valuable for the future where hydrogen gas is being studied as potential biofuel [28].
N3-013	Exiguobacterium undae: According senior research engineer Wei-Min Wu in his report "Biodegradation and Mineralization of Polystyrene by Plastic-Eating Mealworms. 2. Role of Gut Microorganisms", injections of exiguobacterium undae into the gut of mealworms allows them to survive solely on a diet of polystyrene, and thus, can significantly help in the biodegradation and mineralization of plastic [29].
N3-014	Exiguobacterium undae: refer to N3-013
N3-015	Exiguobacterium undae: refer to N3-013
N3-034	Vibrio sp. B2-15-2 16S: is capable of producing biosurfactant that can be applied into environmental, technical, and potentially pharmaceutical industries [30].

A wide diversity of environmentally useful bacterial species was identified in Sulaibikhat Beach. The most abundant was Exiguobacterium undae. Its property of reverting mealworms to feed solely on polystyrene has enormous potential as an eco-friendly way of degrading plastic. Next, Carboxydothermus hydrogenoformans's diet of only toxic carbon monoxide and water, which later produces hydrogen gas, could provide an alterative for oil. Lastly, a useful species of Vibrio, sp. B2-15-2, was identified amongst its pathogenic counterparts and demonstrates the impressive biodiversity of marine bacteria and their respective properties.

4. Conclusion

The data analysis revealed that Kuwaiti water samples harbor extremely diverse bacteria belonging to over 53 bacterial genera. 24 genera were isolated in Salwa Beach, 17 in North West Beach, and 12 in Sulaibikhat Beach. In Salwa Beach samples, identified genera consisted primarily of Alteromonas (17.5% out of 40), North West Beach samples consisted of prominently Marinobacter (26.8% out of 41 sequences), and Sulaibikhat Beach samples consisted of mostly Vibrio (46.3% out of 41 sequences). Alteromonas and Marinobacter strains were found to be abundant and distributed in both Salwa and North West Beach, while none were cultured and identified in Sulaibikhat Beach.

Each sampled location exhibited specific patterns of cultivated pathogen bacterial species that reveals information regarding each beach's unique marine condition and life. In Salwa Beach, most pathogens were tapeworms, only Schistosoma mattheei were isolated from North West Beach, and 3 pathogenic species of Vibrio was found in Sulaibikhat Beach. Since tapeworms and Vibrio species are ubiquitous in aquatic environments copious in marine life, the intense mariculture in Salwa and Sulaibikhat Beach may be partially accountable for the high occurrence of these pathogens.

On the other hand, identified industrially useful bacteria demonstrated no pattern in accordance to sampled location, but similarities in valuable property. While a wide range of diverse, unrelated bacterial cultures were isolated in each beach, the majority of all had biodegradative and biosynthetic capabilities. For example, Sphingomonas found in Salwa Beach can utilize toxic compounds and contaminants as nutrients, Marinobacter hydrocarbonoclasticus found in North West Beach can effectively degrade hydrocarbons, and Carboxydothermus hydrogenoformans found in Sulaibikhat Beach can feed almost entirely on toxic carbon monoxide and water, while producing hydrogen gas. Upon further study, these species-along with the others identified, potentially offer incredible scientific breakthroughs in the usage of microorganisms in pharmaceutical, biotechnological, agricultural, chemical, and environmental fields.

In summery, the purpose of this study was to cultivate, isolate, identity, and determine properties of marine bacterial species in Kuwait from Salwa Beach (N1), North West (N2) and Sulaibikhat Beach (N3) through PCR amplification of 16S rRNA gene, and DNA sequencing of bacteria grown from collected water samples. The findings indicate towards a high level of macrobiotic diversity, and shed light on the ecological functions of both pathogenic and useful bacterial isolates-information that can be used towards precautionary causes or further inquiry and useful application.

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