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New local genetically registration of environmental bacteria isolated from different sources in Basrah city-Iraq

Hala F. Hassan [1*], **Adnan I. Al-Badran** [2]
[1]Department of Marine Biology, Marine Science Centre, University of Basrah, Basrah, Iraq
[2]Department of Biology, College of Science, University of Basrah, Basrah, Iraq

Abstract
Background: *The bacteria was considered one of the main microorganism in the ecosystem particularly, performing numerous functions in the environment, which maintain ecosystem balance, including pollutant degradation and biodegradation of toxins.*
Aim of study: *Soil and water bacterial screening from different locations in Basrah city, Iraq.*
Methods: *Fifty sterile container were used for collection of 50 samples (25 Soil and 25 Water) from different sites of soil and water in Al-Basrah city. DNA was extracted from environmental bacteria using the total DNA extraction Kit following the manufacturer's protocol for Presto™ Mini g DNA bacteria (Genaid). The Basic Local Alignment Search Tool software (BLAST) was used for identification of bacterial species and using the National Center for Biotechnology Information (NCBI).*
Results: *we recorded 13 isolates of bacteria from soil and water in the south Iraqi as a new global species depending on the extraction of DNA followed by 16S rRNA gene PCR amplification and sequencing data analysis. These 13 new isolates were recorded in Basrah province including Staphylococcus gallinarum, Psychrobacter nivimaris, Terribacillus aidingensis, Bacillus cabrialesii, Photobacterium halotolerans, Bacillus paramycoides, Rheinheimera baltica, Staphylococcus saprophyticus, Micrococcus endophyticus, Rheinheimera aquimaris, Zobellella denitrificans, Pseudomonas zhaodongensis, Pseudomonas hydrolytica.*
Conclusion: *Our recorded 13 types of bacteria from soil and water in the south Iraqi. These species were documented as new records in Iraq, while others were index in the National Center for Biotechnology Information (NCBI), the European Nucleotide Archive (ENA), and the DNA Data Bank of Japan (DDBJ) in sequence.*
Keywords: *16S rDNA sequencing, bacterial isolates, phylogenetic tree, Staphylococcus gallinarum, Psychrobacter nivimaris*

INTRODUCTION

Environment is described as the conditions that envelop an organism or a group of organisms. Ecosystem refers to the intricate social or cultural circumstances that influence an individual or community. As humans live in both the natural environment and the constructed, technological, social, and cultural spheres, each plays a significant role in our surroundings (1). Microorganisms are living entities ranging in tiny size from a few millimeters to nanometers and are found in all ecological atmospheres like air, water, and soil. They typically exist as a communication of naturally different collections, engaging with one to another as a groups and competing for the scarce nutrition (2). The water could have different kinds of microorganisms, including both helpful and harmful bacteria (3,4). Tiny organisms in the environment offer numerous ecosystem benefits. They contribute importantly to the decomposition of carbon-based material in the soil, supply nutrition for vegetal development, facilitate the cycling of different elements in biogeochemistry, and help with detoxification .

The methods utilized for identifying bacteria typically rely on culture media and morphological as well as biochemical testing. Conventional identification techniques require a minimum of 3–5 days and demand specialized expertise (5). Molecular techniques, including 16S rDNA (PCR and sequencing) and quality PCR (qPCR), have also been created (6). Bacteria found in various habitats can exhibit distinct phenotypic characteristics, and there are only a few studies that have shown how bacterial habitats influence identification accuracy (7). The significance of bacteria in ecosystems and the environment is crucial, and their isolation, identification, and characterization are essential to meet diverse human requirements (8).

As a result, certain microorganisms like pathogenic bacteria can be detrimental to humans, as they may lead to infections and diseases that can occasionally be life-threatening. Water is regarded as a medium for numerous illnesses. Drinking this polluted water can result in infections from various bacterial and viral illnesses (9). Water is among the five essential elements of the earth (10), and it is vital for the existence of living organisms within the biosphere, as noted by the Central Pollution Control Board (11). Pollution of environmental water is a significant issue impacting rivers, lakes, groundwater, and surface water linked to these locations (12). Overall, the physical, chemical, and biological standards of water have deteriorated because of the introduction of different kinds of chemicals, microbial pathogens, and more (13).

Recent research on water scarcity has highlighted that a substantial bacteriological accumulation in the soil is sustained by a vast array of organic materials in the ground. The majority of these microorganisms are bioactive and thrive in the upper few inches of agricultural soils (14). Microbial agents are capable of existing in various environments alongside humans and can thrive in extreme conditions, including the oceanic crust rocks (15), as well as in cold temperatures or hot springs (16).

Microbial presence in soil relies on ambient conditions influenced by vegetation types, soil texture and chemistry, nutrient availability, pH, moisture, climate, and temperature. Moreover, the soil microbiome offers significant potential for enhancing more eco-friendly farming practices. Soil-dwelling microbes that occur naturally affect plant health, efficiency in resource use, and biocontrol. Nonetheless, their potential has not been fully utilized up to this point .

Additionally, the disposal of organic waste from agricultural lands guarantees the presence of high nutrient levels in the soil, promoting the growth of microorganisms (17). Certain soil bacterial communities, including Rhizobium and Pseudomonas, play a crucial role in nutrition pathway (18). The recent capability to quickly sequence and identify DNA obtained from soil samples has led to the creation of various effective metagenomic analysis methods (19). For instance, examining the inheritances features of entire bacteriological groups enables us to investigate the physiological traits and possibilities of microorganisms linked to plants (20). Modern developments in DNA or RNA nucleotides data base sequence and analysis methods have facilitated and paving the way for novel assay of exploration into soil and water bacterial environments, providing the chance to gain a deeper understanding of, and thus utilize this origin .

METHODOLOGY

Collection of Samples

Fifty sterile container were used for collection of 50 samples (25 Soil and 25 Water) between December of 2023 to March of 2024 aseptically in a volume of 100 mL capacity from different sites of soil and water in Al-Basrah city. Each container have specific number, collection date and collection area then transported to the Microbiology Laboratory in College of Science, Biology Department.

Sample preparation

The preparation of samples utilized a classical method which is quick and straightforward, utilizing bacterial colony isolated directly from pure culture media with sterile inoculating loops. All culture plates, including Nutrient Agar (NA) and Broth Agar (BA), were made depending on the manufacturers' guidelines and autoclaved at 121°C for 20 minutes. The technique was used for DNA extraction from bacterial cells utilizing special DNA kit extraction (21) .

Total count (serial dilution)

Six falcon tubes with 9 ml of distilled water were autoclaved for sterilizing at 121 ^{o}C for 15 min and the first tube was incubated with one ml of water sample which is taken from 100 ml of sample by (100-1000) µl micropipette. Subsequently, drawing 1ml from each 9 ml of the tube sample number one and mixed with the second 9 ml tube to be 10 ml and again take 1 ml to mix with other, and this process continued until the sixth tube once will be 10 ml, using sterile micropipette. One hundred µl from each tube was taken by (100-

1000) µl micropipette and cultured on Nutrient agar then incubated at 37° C for (24-48 h). The colonies of N.A. was calculated and multiplying with dilution factor to estimate the number of bacterial cell in one ml of water with disregarding the dishes which have less than 30 and more than 300 colony (22). Then determine the acceptable level of coliform in water according to formula in the review of Barrell *et al.*, as below (23).

No. of bacterial cells in one ml = The number of colonies on agar plate × 10^{-1} × dilution factor

Molecular Identification

Extraction of DNA

DNA was extracted from environmental bacteria using the total DNA extraction Kit following the manufacturer's protocol for Presto™ Mini g DNA bacteria (Genaid). In summary, a loopful of pure colonies was collected from the Nutrient Agar plate's surface and refer to the Kit procedure leaflet with serial number L126505, these colony should place into 5 mL of sterilized nutrient broth then incubated overnight at 37 °C.

Identification of isolates using PCR

PCR was conducted to verify the identity of the bacterial isolates by detecting the 16S rDNA gene universal primer (Table 1), as described earlier (24).

Table 1: The universal primer 16S rDNA sequence and product size

Primers	Sequence of primers	Length
27 F	5-AGAGTTTGATCCTGGCTCAG-3	20 bp
1492 R	5-GGTTACCTTGTTACGACTT-3	19bp

All the experiments of PCR were performed using a standard PCR machine (Applied Biosystem). The gene was amplified employing 25 µL of each PCR component along with 2 µL of the DNA template. The total volume was adjusted to 19 µL by adding double distilled water and including 2 µL of each primer. Amplification involved an initial denaturation at 95°C for 5 minutes, then a denaturation at 95°C for 30 seconds; primers annealed at 55°C for 30 seconds and extension occurred at 72°C for 1 minute. The last extension took place at 72°C for 5 minutes, and the complete reaction was carried out for 37 cycles. The PCR products were amplified and analyzed using electrophoresis on a 1.5% agarose gel at 70v (Power supply, Fisher Scientific, UK) for 1 hour, stained with Ethidium bromide, visualized using a UV Transilluminator, photographed, and subsequently stored at -20 °C until being sent for sequencing (25). The mixture was centrifuged and vortexed for 1 minute; the PCR reaction program is outlined in (Table 2).

Table 2 : Reagent (50 µl) of PCR for amplifying 16S rDNA gene

Reagent	Volumes (µl)
Go Taq green master mix	25
DNA template	2
Primer forward	2
Primer reverse	2
Nuclease free water	19
Total volume	**50**

Sample preparation for sequencing

A total of 25 µl of PCR product for 16S rRNA gene was dispatched to Macrogen company in South Korea for sequencing. Each sample's PCR product was assigned a label matching the corresponding number in the Excel sheet provided by Macrogen, and the company carried out the purification of the products for sequencing and the data base analysis as well (26).

Bacterial species identification

The Basic Local Alignment Search Tool software (BLAST) was used for identification of bacterial species and using the National Center for Biotechnology Information (NCBI) as approved by Kerbauy *et al.* for registering in Gene bank (27). The nucleotide sequence was copied and pasted into BLAST after proofreading, allowing the program to identify the bacterial species by comparing their sequences with other reference strain.

Phylogenetic Tree and Diversity Relationship

A phylogenetic tree is a visual model similar to a tree that depicts the evolutionary and phylogenetic connections among biological taxa based on their physical or genetic traits, also referred to as a cladogram, tree of life, or evolutionary tree (28,29). In constructing the Neighbor Joining Tree, all acquired 16S rRNA sequences were trimmed based on sequencing quality. Multiple cluster alignment and phylogenetic analysis were executed using MEGA 11 software (Molecular Evolutionary Genetics Analysis) Version 11, applying the neighbor binding method with a 1000-cycle bootstrap to assess statistical support (30,31) .

RESULTS AND DISCUSSION

The DNA extracted from all samples underwent gel electrophoresis (Figure 1) and was used to amplify the 16S rRNA gene via PCR.

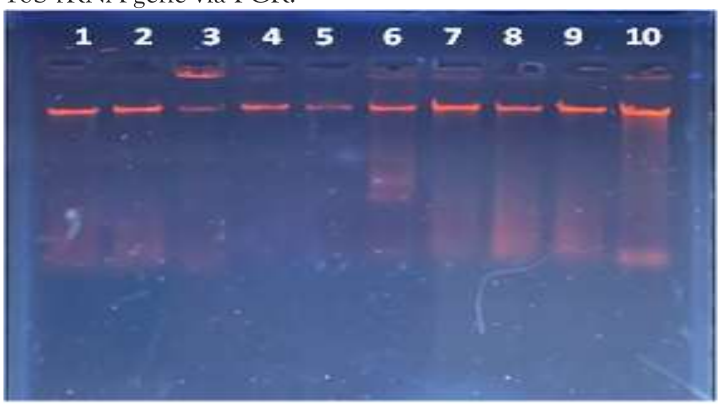

Figure 1 : Agarose gel electrophoresis (0.2gm) showed a model of Genomic bacterial isolates DNA Lane 1-10: bacterial isolates DNA bands

The enhanced bands of every bacterial isolate were observed under UV transillumination, revealing a single band for each isolate approximately 1500bp in size when contrasted with a standard ladder (Figure 2). The 16S rRNA gene from 36 isolates was effectively sequenced, and the bacterial species were identified following trimming and processing with (BLAST). The optimal dilution for achieving single colonies is $^{10-2}$ with water samples and $^{10-6}$ for soil samples. The origin of these bacterial strains included: seven strains obtained from soil and six strains obtained from water. Our ongoing study comprised fifteen samples gathered from various locations. The origins of these bacterial strains were: seven strains extracted from soil, six strains extracted from water, and known types of bacteria from the environment were collected. In contrast to other researchers who documented *Bacillus paramycoides* strain in soil contaminated with oil (32), besides, these findings align with the information provided by Zhang *et al.* (33), who confirmed in their research that *Pseudomonas zhaodongensis* sp was documented. Comparable to a different study conducted by Li *et al.* (34).

16S rRNA gene Data Analysis

For the 16S rRNA gene sequences of the isolated bacterial species, a rooted phylogenetic tree was generated (Figure 2). The tree was drawn using Mega 11 program and after concatenated of the sequences for all subjected isolates. The phylogenetic tree showed similarity between 2 isolates of Bacillus, Staphylococcus isolates, 2 Rheinheimera isolates , 2 Pseudomonas.

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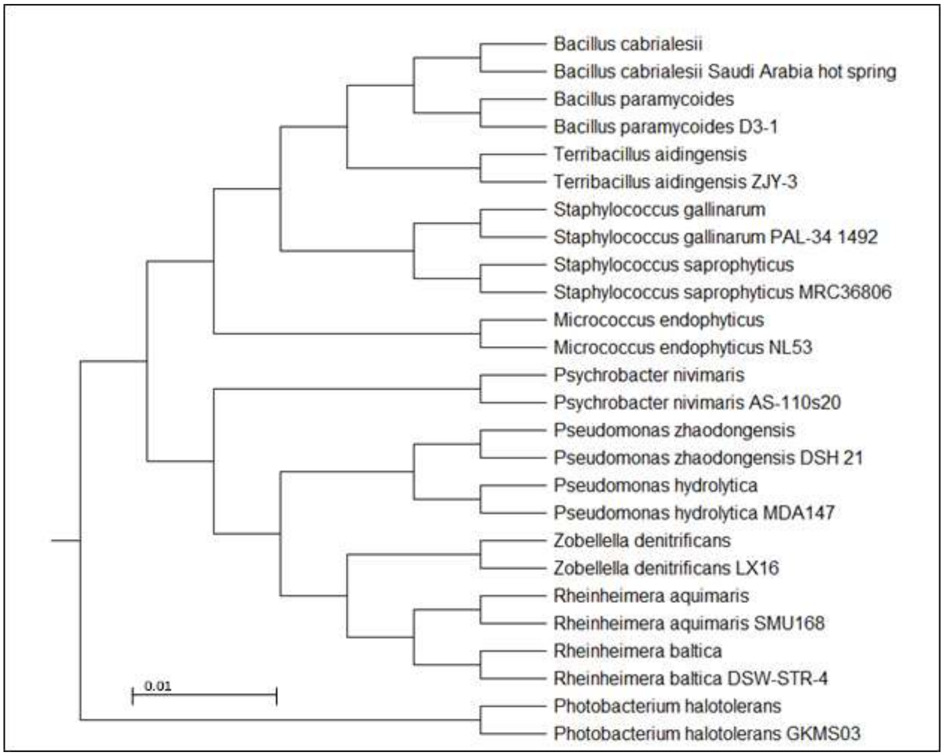

Figure 2 : Rooted Neighbor Joining phylogenetic tree constructed using concatenated sequences of 16S rDNA gene for isolated bacteria during the present study this Neighbor Joining tree showing the distribution and relationships of 13 different bacterial isolates from soil and water that is constructed from MEGA 11 software algorithm

Morphological and Molecular Identification of Bacterial isolates

(A): Morphological identification: The phenotypic traits of the colonies developing on nutrient agar medium, for every strain, were described. According to Gram-staining, the findings indicated that 6 of the isolates were Gram-positive, while 7 were Gram-negative. (B): Molecular identification involved DNA extraction and amplification analysis of 16S rRNA. The electrophoresis results for genomic DNA extraction indicated pure and clear isolated DNA for all isolates, which were then used for 16S rRNA sequencing. Moreover, all isolates underwent testing for the specific amplification of 16S rRNA gene sequences utilizing a set of universal primers, 27 F and 1492R (Table 1), resulting in a single amplification of approximately 1500 bp for all isolates (Figure 3).

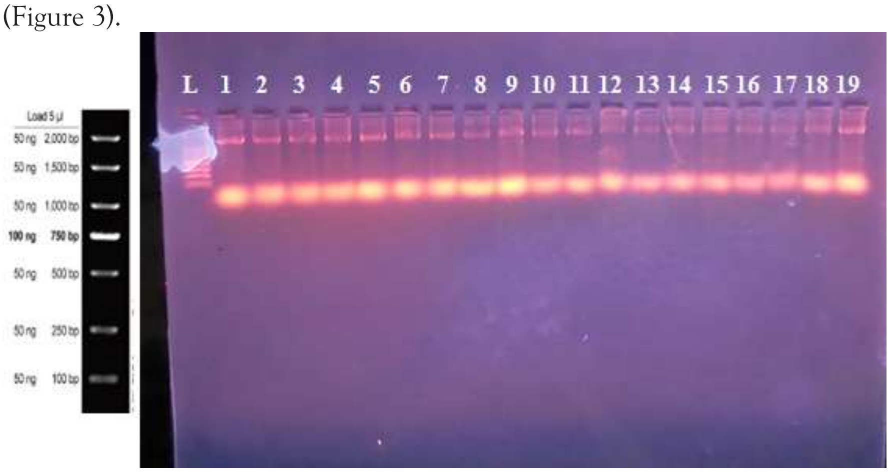

Figure 3 : Agarose gel electrophoresis (2%) showed a model of amplified 16S rRNA gene (1500bp). Lane L: 100bp-2Kbp Ladder, Lane 1-19: 16S rDNA gene bands for bacterial isolates

Derived from the partial sequencing of the isolated samples obtained. The recent isolates (newly recorded isolate in Basrah province) include Staphylococcus *gallinarum, Psychrobacter nivimaris, Terribacillus aidingensis, Bacillus cabrialesii, Photobacterium halotolerans, Bacillus paramycoides, Rheinheimera baltica, Staphylococcus saprophyticus, Micrococcus endophyticus, Rheinheimera aquimaris, Zobellella denitrificans, Pseudomonas zhaodongensis, Pseudomonas hydrolytica*. Identifying an appropriate classification and generalization for Bacillus spp. bacteria is challenging, if not impossible, due to their extensive diversity in physiological traits, which enables these species to inhabit nearly all natural environments such as soil, air, lake sediments, and water, as well as extreme conditions like acidic hot springs, salt marshes, and sub-Antarctic soils; Bacillus species' abundance has been noted (35,36). Following identification and treatment via (BLAST), thirteen strains were documented in Basrah province as shown in (Table 3).

Table 3 : Identified bacterial isolated from different sources, soil and water also Gram stain

Strain name	Gram stain	Source strain
Staphylococcus gallinarum	+ve	Soil
Psychrobacter nivimaris	-ve	Water
Terribacillus aidingensis	+ve	Soil
Bacillus cabrialesii	+ve	Soil
Photobacterium halotolerans	-ve	Water
Bacillus paramycoides	+ve	Water
Rheinheimera baltica	-ve	Water
Staphylococcus saprophyticus	+ve	Water
Micrococcus endophyticus	+ve	Soil
Rheinheimera aquimaris	-ve	Water
Zobellella denitrificans	-ve	Soil
Pseudomonas zhaodongensis	-ve	Soil
Pseudomonas hydrolytica	-ve	Soil

CONCLUSION

Collecting and isolating bacteria from different environments allows us to register and discover a new bacterial strain in soil and water that may provide benefits. Using various culture and molecular methods lead to identify 13 new isolates of bacteria from soil and water in southern Iraq. The findings based on the 16S rRNA gene sequence revealed that the isolates were grouped into different proportions depend on their origin. Some species were noted as a new global record in Iraq, while others were documented in the National Center for Biotechnology Information (NCBI), the European Nucleotide Archive (ENA), and the DNA Data Bank of Japan (DDBJ) in that order, along with phylogenetic analysis from the taxonomic classifications as first time we registered in Iraq which isolated from the water and soil.

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Conflict of interest
The authors have stated that there is no conflict of interest present.

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