Molecular detection of new Bacillus strains from soil samples of free grazing areas in Basrah province, Southern Iraq

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Abstract: Bacterial contamination is an important indicator for soil quality that could be related to the plant's suitability for animals 'grazing and ultimately human public health. Consequently, the microbial characteristics of soil could be linked to animals' products quality, such as milk and meat. Bacillus species is one of the most distributed soil contaminating bacteria and is linked to animals' products quality. Three different grazing areas (Abo Al-Kasib, Al-Seba, and Al-Fawdistricts) in Basrah province were selected to determine the bacterial (Bacillus)distribution in soil. Samples were randomly collected from each field of the study. Based on the bacterial cell shape and staining (microscopy), colony morphology, biochemical tests, and the 16S rRNA genes amplification results, the bacterial isolates were identified to be from the genus Bacillus, of which *Bacillus cereus* was dominant in all samples. Five new isolates were identified based on 16S rRNA nucleotide sequences. Phylogenic analysis showed a close relation between three isolates to *Bacillus cereus*with high 16S rRNA gene sequences similarity 99% (SAMU1), 99% (SAMU2) and 98.9% (SAMU3). Furthermore, two isolates showed sequences similarity to *Bacillus paramycoides* (99%, SAMU4) and *Bacillus safensis* (99%, SAMU5). All the above isolates were registered in the NCBI centre under the following names *Bacillus cereus* strain SAMU1, *Bacillus cereus* strain SAMU2,*Bacillus cereus* strain SAMU3,*Bacillus paramycoidess* strain SAMU4 and *Bacillus safensis* strain SAMU5 and were assigned the following accession numbers MK418732, MK490900, MK490901, MK490902,and MK490903respectively.In addition, some isolates were found to be 100% similar to the already identified Bacillus strains such as *Bacillus safensis, B. pummilus, B. paramycoides* and *B. safensis*. The current study investigated and mapped the distribution of Bacillus Spp in animals grazing areas that had been subjected to salinity increment.

Keywords: Bacillus, Bacillus cereus, Grazing field, Soil

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1. Introduction

The soil is considered as one of the richest bacterial environments for isolation of bacillus spp. Bacillus genus was described for the first time by Cohnin 1872 [1]. This discovery was followed by describing several hundrednovel species.Bacillus species are rod-shaped, Gram-positive, motile, endospore-forming, and chemoheterotrophic bacteria. They are catalase positive and facultative anaerobic or aerobic [2].Most of Bacillus genius members are characterized by the high level of physiological capabilities and spore formation, which help them to grow in wide range of environments [3].Some Bacillus species members are considered as an important soil contamination factors in many countries [4-6]. Among all Bacillus species, *Bacillus cereus* was found to be a distinct family member that plays a big role in soil contamination [7,8]. It is believed that this microorganism contaminates animal products such as milk at unhygienic milking procedure, and meat during slaughter which decreases the shelf life of these products [9].Furthermore, it is frequently found in vegetables and can be found in food [10,11]. Strains of this genus are able to produce specific toxins that lead to two types of food poisoning with common symptoms either vomiting or diarrhea[9,12,13].The current study aimed to determine the soil bacterial pollution and to identify Bacillus species in soil samples related to free grazing areas, which has a direct relationship to the animal's products hygiene and ultimately human health.

2. Material and Methods

2.1 Sample collection

Fifty soil samples were collected from three different grazing areas from Al-Basrah province, including Abo Al-kaseb, Al-Seba,and Al-Faw throughout the 6weeks of the study period which started from August 2017. Approximately 5 g of samples were collected from 5-10 cm depth below the soil surface and placed in adry, clean, and sterile polyethylene tube. Seventeen samples from each area(except sixteen samples from Abo Al-kaseb) were collected. Directly after collection, all samples were transported aseptically to the microbiology laboratory of the college of sciences, Basrah University; under sterile conditions. Samples were processed directly or kept at 4ºC until they were used [14].

2.2 Geographic location

The three selected regions are located at the second half of the river Shatt Al-Arab. This river is approximately 204km in length, starts at Al-Qurna city, Basrah southern Iraq, where the Tigris and the Euphrates rivers confluence. Passing throw many districts southeastern, the final destiny of Shatt Al-arab is the Arabiangulf. Al-Faw is the nearest city to the sea, whereas AboAl-kaseb is about 100km far from the sea, while Al-seba is located between them figure (1).

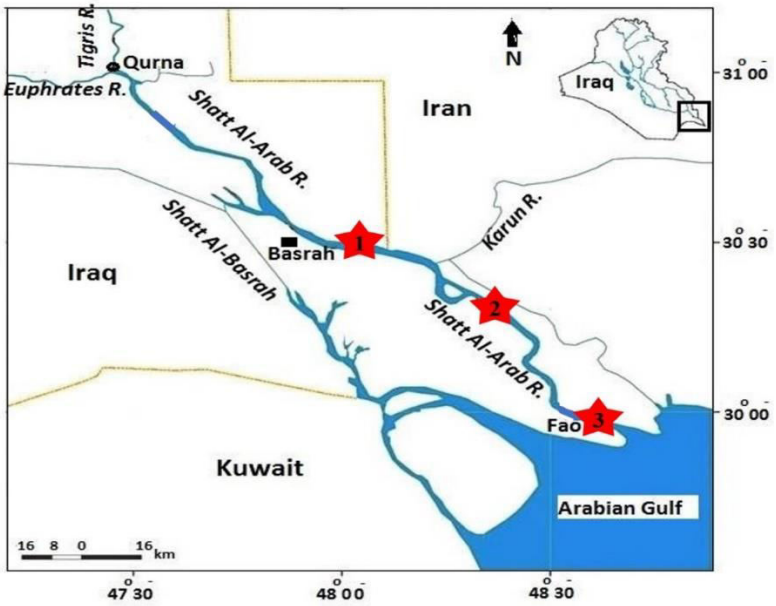

Figure (1): Map of the river Shatt Al-arab and the districts around it (Mohamed and Abood, 2018). The location of the fields of study were labeled as red stars, number 1 is Abo Al-Kaseb, 2 is Al-Seba and 3 is Al-Faw.

2.3 Isolation of *Bacillus spp*.

One gram of each of the soil samples was suspended in nine ml sterile distilled water (DW) forming stock solutions. Ten sterile 15ml tubs containing 9 ml Distilled water were used to form serial dilutions for each of the sample's stocks. The dilutions were made by transporting 1ml from the prior concentration to the next one until reaching 10-10 then streaking on Nutrient agar, Blood agar, and Tryptic soy agar and incubated at 37oC for 24 hours [15].

3. Identification of Bacterial Samples

3.1 Morphological:

Initial diagnosis of isolates depended on the morphological characteristics of the colonies which includes colony shape, colony color, texture and edges [16].

4. Molecular study

4.1 DNA extraction

The DNA extraction was conducted from (19) samples that gave morphological characteristics of Bacillus spp. Suspected Bacillus isolates were grown in Muller Hinton broth for 18 hours and harvested by centrifugation at 5000rpm for 4 minutes at room temperature. The supernatant was discarded out and the pellets were used to extract the genomic DNA (gDNA) by

using a commercial kit (Presto Mini DNA Bacteria kit, Geneaid, USA), following the manufacturer's protocol. Quality and quantity of the extracted gDNA were obtained by measuring the absorption at 280nm and 260nm in nanodrop. Then samples from the isolated gDNA were ran on 0.8% agarose electrophoresis gel to confirm the DNA mass quality and quantity.

4.2 Detection of 16S rDNA

A pair of universal primers forward (27 5'-AGAGTTTGATCCTGG-3') and reverse (1492 5'-GGTTACCTTGTTACG-3') were used to amplified 16S rRNA genes from the isolated gDNA [18,19] . Each reaction tube contained 2µl (20µg) as gDNA template, 2 µl (20 pmol) forward primer, 2 µl (20 pmol) reverse primer, 25 µl master mix, and the final volume was adjusted with free nuclease water to 50µl. Polymerase chain reactions were started by an initial denaturation temperature 95^{o}C for 5 minutes followed by 35 cycles of denaturation temperature 95^{o}C for 30 seconds, annealing temperature 56^{o}C for 30 seconds and extension temperature 72^{o}C for 1 minute, then ended with a final extended temperature 72^{o}C in 10 minutes and hold at 8^{o}C. The amplicons were loaded into 1.5% agarose gel and were ran for 25 minutes. The results of PCR bands were stained with ethidium bromide and visualized using a UV transilluminator.

4.3 Purification and Sequencing of 16S rRNA

The resulting PCR Products were purified with a QlAquick PCR purification Kit (Qiagen,USA).The purified PCR products were sent to macrogen company, Korea, then aligned with known16S rDNA sequences in Genbank using the basic local alignment search tool BLAST www.blast.ncbi.nlm.nih.gov/Blast.cgi.Sequence with ≥99% similarity was used for the identity diagnosis [20] . Phylogenetic tree was constructed by using Mega 6 program.

5. Results and Discussion

This study was conducted to understand the microbial contamination in soils associated with animals grazing and its possible impact on public health. Bacillus is one of the widely distributed soil contaminants and its presence can be used as an indicator for soil quality for animals and public health. Due to the ability of Bacillus spores to resist conventional pasteurization procedures, milk and milk products can be affected negatively by these bacteria especially under poor preservation conditions [20]. Some members of the Bacillus species are known to cause food poisoning outbreaks, and this amplifies the need for understating the epidemiology of this species and factors promoting its risks to public health. It has been shown that salinity toleration is one of the predisposing factors for Bacillus distribution [21] . Therefore, we investigated the identity and distribution of Bacillus bacteria in grazing areas that had been affected by sharp increment in salinity in 2017. To our knowledge, there are limited studies that detected Bacillus bacteria in the areas around the second half of the river Shatt Al-Arab. Compared to our study, these studies selected only one region, such as Al-Seeba district [22] or Al-Faw district [23].Whereas Shareef et al. investigated Abo Al-Kaseeb district using water samples. Furthermore, there is no information about Bacillus bacteria in these regions duringthe salt rising crisis (2017-2018), particularly in grazing areas. Due to the importance of these districts (Abo AlKaseeb, Al-Seeba, and Al-Faw)for animals' feeding, they were selected as free grazing fields. To achieve nonbiased selection, we collected samples randomly from the selected grazing fields. This study depended Bergey's manual of systematic bacteriology [24] for identifying morphological characteristics and biochemical test, then detection of 16SrRNA techniques was used to confirm the identification.

5.1 Phylogenetic tree

Result of nucleotide sequences for the 19 Bacillus isolates were concatenated to produce a sequence length 689 base pair depend on the shorter Sequence among the species. Evolutionary history was inferred using the unweighted pair group method with arithmetic mean method (UPGMA). A phylogenetic tree was constructed based on 16S rRNA genes sequencing and some reference strains and displayed in figure (2).

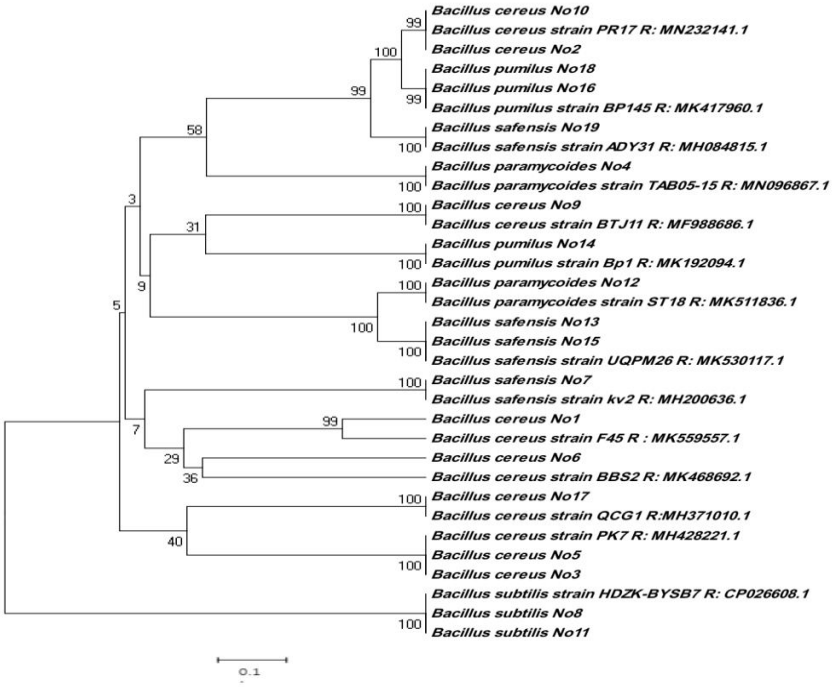

Figure (2) The evolutionary history was inferred using the UPGMA method, concatenated sequences of 689 base pair for each strain derived from an alignment of 16S rRNA gene sequences. The evolutionary analyses were conducted in MEGA6 showing the distribution and phylogenetic relationships between 19 *Bacillus sp.* isolated from grazing area in this study and 14 reference strains (R). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates)

5.2 Identification of new strains

Five bacterial isolates (No.1, No.6, No.17, No.12 and No.7) were different from reference strains at only one to four nucleotide sequences. These isolates were recognized as new isolates. Therefore, they were submitted to the National Center for Biotechnology Information (NCBI)and recorded in theGenBank for DNA database sequences. *Bacillus cereus* strain SAMU1 (isolate No.1), *Bacillus cereus* strain SAMU2 (No.6), *Bacillus cereus* strain SAMU3 (No.17), *Bacillus paramycoides* strain SAMU4 (No.12) and *Bacillus safensis* strain SAMU5 (No.7) with GenBank:MK418732.1, GenBank:MK490900.1, GenBank:MK490901.1, GenBank:MK490902.1 and GenBank:MK490903.1 accession number respectively. The specific mutations are shown in table (1) and figure (3). SAMU1 isolate was found to be 99% identical to *Bacillus cereus* strain F45 with some point mutations including, C deletion in position 106, replacement of G by A, G by T in position 113, 124 respectively and an insertion of G in position 123. The SAMU2 isolate was closely related (99%) to *Bacillus cereus* strain BBS2 with some point mutations that included, deletion of T at position 37, and replacement of A by T, G by A and T by G at position 39,40 and 136 respectively. The SAMU3 strain (GenBank:MK490901.1) is highly identical (99%) to *Bacillus cereus* strain with only one difference at position 29 where G is replaced by A. Only two nucleotides differences were recorded between SAMU4 strain and *Bacillus paramycoides* strain ST18 at position 92, 394 where C and G are replaced by A and C respectively. Finally, high identity (99%) were found between the SAMU5 strain and *Bacillus safensis* strain Kv2 with only one-point mutation at position 24 where A is replaced by G table (1) figure (3). These differences in the genetic makeup of the isolated strains are probably referred to adapt to the changing environment in the studied geographical area in the current study. The environmental changes and salinity in southern Iraq are persistent problems that drive bacterial communities to evolve molecular stress response mechanisms. Whether these mutations have a specific role in the tolerance and survival of the isolated strains is currently not known. It would be important to relate these genetic differences to the physiological processes to overcome salinity stress.

Table (1): The new global strains with type of point mutation and location in the sequences

Isolate	Closest Strain	Identity	Sequence differences	Nucleotide	Location
SAMU1	*Bacillus cereus*	99%	Deletion	C	106
			Replacement	G by A	113
				G by T	124
			Insertion	G	123
SAMU2	*Bacillus cereus*	99%	Deletion	T	37
			Replacement	A by T	39
				G by A	40
				T by G	136
SAMU3	*Bacillus cereus*	99%	Replacement	A by G	29
SAMU4	*Bacillus paramycoides*	99%	Replacement	C by A	92
				G by C	394
SAMU5	*Bacillus safensis*	99%	Replacement	A by G	24

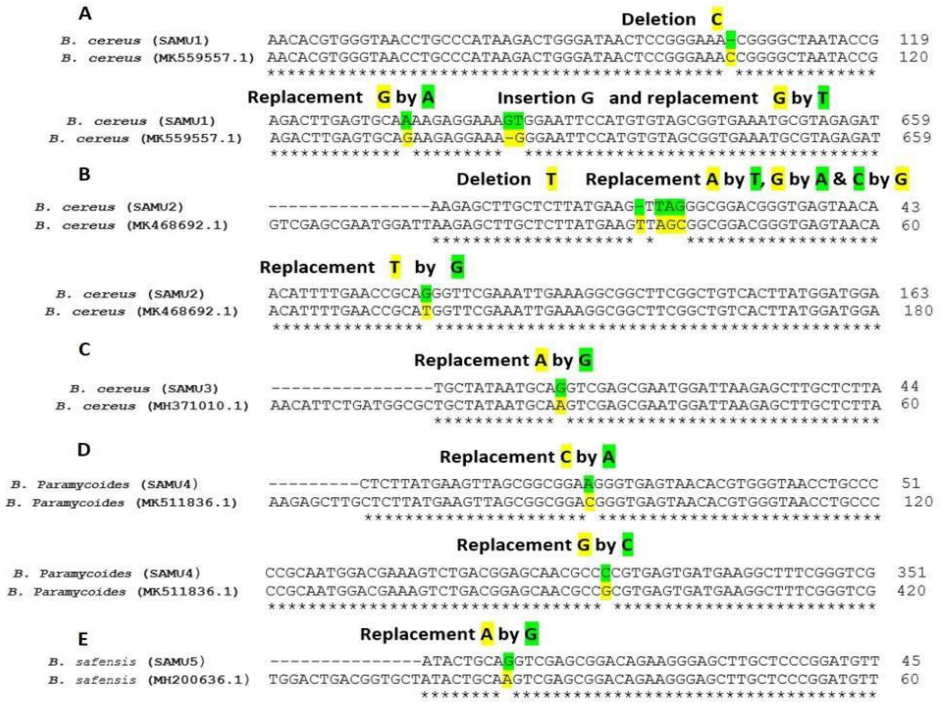

Figure (3): Sequences alignment of 16S rRNA of the isolated Bacillus strains against some standard isolates. The differences between isolates sequences were highlighted.

The data showed that among 50 soil samples, 71isolates of different types of bacteria were isolated, only 19 isolates were identified as Bacillus spp. Eight of the isolates were found in Abo Al-Kaseebarea. They were classified into five species; *Bacillus cereus* 3 (10 %),*Bacillus safensis* 2 (6.67%), *Bacillus pumilus* 1 (3.33%),*Bacillus subtilis*1 (3.33%) and *Bacillus paramycoides*1(3.33%).Furthermore, 6 Bacillus isolates were found in Al-Sebaregion, which subdivided into five species as well; *Bacillus cereus* 2 (8.69 %),*Bacillus pumilus* 1 (4.35%), *Bacillus safensis*1 (4.35%),*Bacillus subtilis*1 (4.35%) and

Bacillus paramycoides 1(4.35%). In addition,5 isolates in Al-Faw are classified into three species *Bacillus cereus* 3(16.7%),*Bacillus pumilus* 1 (5.55%), and *Bacillus safensis* 1 (5.55%) table (2).

Table (2): description of the collection region, number of samples and distribution ratio for the isolated bacteria.

No.	Collection region	Samples No.	Isolates No.	Distribution & (%)	Type of isolates
1.	**Abo Al-Kaseb**	**16**	**30**	**1 (3.33)**	***Bacillus pumilus***
				3 (10)	***Bacillus cereus***
				1 (3.33)	***Bacillus subtilis***
				2 (6.67)	***Bacillus safensis***
				1 (3.33)	***Bacillus paramycoides***
				22 (73.33)	**Other species of bacteria**
2.	**Al-Seba**	**17**	**23**	**1 (4.35)**	***Bacillus pumilus***
				2 (8.69)	***Bacillus cereus***
				1 (4.34)	***Bacillus subtilis***
				1 (4.34)	***Bacillus safensis***
				1 (4.34)	***Bacillus paramycoides***
				17 (73.91)	**Other spesies of bacteria**
3.	**Al-Faw**	**17**	**18**	**1 (5.55)**	***Bacillus pumilus***
				3 (16.7)	***Bacillus cereus***
				1 (5.55)	***Bacillus safensis***
				13 (72.2)	**Other species of bacteria**
Total		**50**	**71**	**71 (100)**	**All isolates**

The similarity of occurrence of some of the isolated strains in all the regions of the study might be related toone or more of the following reasons. 1) soil salinity: even though there were long distances between the three soil regions used to collect samples in this study (Abo Al-kaseb to Al-seba =30km, Al-Seba to Al-Faw =50km), all of these areas are supplied by the river Shatt Al-Arab. During sample collection time, there was a water crisis and the level of the clean water at the river Shatt Al-arab was too low. Consequently, the sea water invaded the river raising the water salinity and leading to an increase in the soil salinity in these districts. It has been shown that Bacillus strains are able to survive in a salty environment and probably dominates in soils compared to other species of bacteria [25].2) Type of growing plants: all the three regions are known for growing palm date trees and grass which might have influenced the variety of Bacillus isolates in these soils. A relationship between type of plant and bacteria was recorded by some studies[9,26].3) Soil compositions and pH: It is well established that soil bacterial communities are influenced by environmental changes including minerals and organic elements in the soil [27].These chemicals include organic carbon, nitrogen, potassium, calcium, magnesium, phosphorous and iron[27].

Bacillus genus includes pathogenic and non-pathogenic strains. *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus subtilis* are important for public health. In the current study, *Bacillus cereus* had the highest frequency of isolated strains. It is one of the common pathogenic members of the bacillus genus. The *B. cereus* was recognized, in the second half of the twentieth century, as a food contaminant which can cause food poisoning [28]. Recently *B. cereus* has been reported as a common etiological factor of localized eye and wound infections in addition to systemic infection [29].The way of transmission of the *B. cereus* to humans is mainly through food chain contamination such asfood and animal products .*B. cereus* was found to be associated with plants in an endophytic relation as an alternative niche [30,31].While *B. cereus* is a common cause for food-borne cases, other Bacillus spp. such as *B. subtilis* is known as food spoilage bacteria. However, *B. subtilis* can cause food-borne illnesses and produce toxins [32]. Furthermore, the *Bacillus pumilus* is rare in human infection, nevertheless, in 2007 a report summarizing 3 case studies was published and concluded that a strain of *Bacillus pumilus* was responsible for

the development of cutaneous lesions similar to those caused by *Bacillus anthracis* [33]. In contrast*Bacillus safensis* and *Bacillus paramycoides*are not recorded as pathogenic species in human and animals.

Bacillus bacteria can improve soil quality and thus improve the plant growth and health [34]. As growth-promoting agents, many Bacillus strains (such as *B.safensis*) were used to improve growth of soybean, wheat and corn [35,36]. Recent study by Cao *et al.* reported a significant effect of Bacillus strains against phytopathogens. However, it is imperative to decrease Bacillus bacterial load in animals and food products to reduce risks to public health [38]. Many studies aimed to understand the factors influencing Bacillus epidemiology and proposed measures to reduce Bacillus transmission to food chain. However, Bacillus epidemiology is not fully understood. This study focused on molecular mapping of the Bacillus spp in selected local grazing areas and highlighted the effect of the environmental changes on the genetic variation and distribution of members of this species. These findings can be related to the total bacillus species germ load in animals' products from these local areas which are commonly do not meet pasteurization and hygiene standards [39].

6. Conclusion

Bacillus soil contamination in grazing areas is detrimental factor for human health and animal's products quality. Bacillus species is capable to overcome salinity and the fluctuating environmental conditions which impose further veterinary and public health challenges.

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