Seasonal variations of Environmental parameters and their effect on the Brucella species in Iraqi animals soil

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Abstract
The soils samples collected from animals soils from of different villages for the fourth season from middle,south and north of Babylon province, Iraq. for the fourth season from winter in February to Autumn in November, Theses samples included 10 sites in Winter, 32 sites in Spring,9 sites in Summer and 12 sites in Autumn, 2021. The samples was taken from the upper layer for bacteriological study and from the deepest layer (25-30 cm in depth) of the soil for environmental study, dried by air and sieved (to ~ 2 mm particle size) during the collecting the samples measured air temperature and soil temperature. This investigation aim to knowledge presence types of *Brucella* spp. in the animal's soils. *Brucella* is a gram-negative, intracellular, non-motile, none sporulating, non-toxigenic, no fermenting, and weakly acid-fast bacteria. The bacteria isolated from soil by Brucella agar base, PCR technique was used to diagnose the genus of Brucella and the gene used 16SrRNA. The results showed the presence of brucellosis in the soil and its percentage varies according to the seasons, where the highest percentage of brucellosis was in summer and the lowest in winter, and these percentages were related to seasonal environmental indicators. The presence of the most Brucella had the highest values of physical and chemical properties (Air and Soil temperature, pH, E.C TDS, Salinity, TOC) except for Toc is lower, while the percentages of the lowest *Brucella* had the lowest environmental indicators except for Toc. It was also noted that this low percentage of Brucella in the winter had the highest values of heavy elements (Cu, Fe,Cd) except for Pb has the lowest value, while the higher percentages in summer had the lowest value (Cu, Fe,Cd) except for Pb being high. The results revealed presence the bacteria in some the soil isolated samples and after senting these samples to DNA sequence noticed different species of bacteria. DNA sequencing for (*B.melitensis, B. pseudogrignonensis, B.lupini* and *B.inopinata*) strains include substituation mutation (transversion and transition) and at compared these results for genotype with phenotype that include (Air and Soil temperature, pH, E.C TDS, Salinity, TOC) by statistical analysis that proportion of bacteria presence in summer is higher and the relation with environmental parameters include (Air and Soil temperature, pH, EC, TDS and Salinity) is high, except TOC is low while proportion of bacteria presence in Winter is lower while bacteria presence in winter is lower. This indicates to presence a relationship between the *Brucella* percentage with environmental parameters and heavy metals according to seasons.
Keywords: Iraqi animal's soil; Environmental parameters; Brucella species

1. Introduction

Ochrobactrum has been recognized as an emerging pathogen in immunodeficient and immunocompetent patients. Recently, the genus Ochrobactrum was renamed within Brucella. Thses bacteria included Ochrobactrum anthropi (newly named B. anthropi), O. intermedium (B. intermedium), O. tritici (B. tritici), O. haematophilum (B. haematophilum) and O. pseudogrignonense (B. pseudogrignonensis) have been reported to cause human infections1. Microorganisms have a varied habitat in the soil, but their numbers are quite high in the surface soil around macrospores2. The macrospores are soil channels that are created by earthworms, plant roots, and other soil biota, and are lined with organic materials in top soil3. Bacterial growth and diversity are connected with organic matter; therefore, microbial numbers are highest at the soil's surface (10 cm) and decrease with depth4. Niche is defined as the "interrelationship of a species and its relational position in a given ecosystem, including the species' connection with the ecosystem's components", As a result, the niche of a species in a given ecosystem can be influenced by all of the components in its ecosystem, and the niche of a species in a particular ecosystem can help set up the aspects of its environment, which are critical for its survival5.

A zoonosis is any disease or infection that is naturally transmissible from vertebrate animals to humans. More than 60% of human infectious diseases are caused by zoonotic pathogens which have been responsible for some of the most fatal diseases such as Ebola and severe acute respiratory syndrome (SARS) in recent years6,7. Zoonotic diseases may be obtained or transmitted in a variety of ways including

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direct contact, through the air (aerosol), contact with an inanimate object that harbours the disease (fomite transmission), oral ingestion and arthropod bite8. Bacterial Zoonoses are diseases that infect millions of people every year, because of food borne zoonoses caused by different types of pathogenic bacteria such as Salmonellosis, Campylobacteriosis, erysipelas, leptospirosis, listeriosis, tuberculosis, anthraz, Brucellosis, plague, Shigellosis, Tularaemia9. Diseases of animal origin can be transmitted between humans and animals through direct contact, indirect environmental contact, and/or through food consumption10. The disease is widely prevalent and has recently been reported in domestic animals and humans in Oman, Qatar, Kuwait, Saudi Arabia, Iraq, Russia, Turkey, Pakistan and the UAE, which share a vast border with Iran and have close economic relationships11. Brucella is a genus with twelve species that infect various wildlife and domestic animal species12. Brucella abortus (cattle), B. melitensis (goats and sheep), B. ovis (rams), B. canis (dogs), B. suis (pigs), and B. neotomae (pigs) are the six Brucella species classified according to pathogenicity and preferred hosts (Common voles, desert wood rat). B. melitensis, B. suis, and B. abortus are the three most important pathogenic species in humans 13. The most important representatives of the genus are B.melitensis, B. abortus, and B. suis, which cause human disease and major economic losses in animals 14. B. canis, B. neotomae, and B.ovis all have lower or non-zoonotic potential, according to Suárez-Esquivel et al.,15. Brucella papionis, Brucella microti, Brucella inopinata, and Brucella vulpis have all lately been detected in aquatic mammals (such as Brucella pinnipedialis and Brucella ceti) and other wildlife (such as Brucella papionis, Brucella microti, Brucella inopinata, and Brucella vulpis) 16. It is one of the most important infectious diseases that is transmitted to humans either by direct contact with infected animals or through the consumption of contaminated animal products, particularly unpasteurized milk and soft cheese 17. According to international reports, the largest incidence of Brucellosis (Malta fever) is seen in Iraq, Syria, Turkey, Mongolia, and Kyrgyzstan18. Brucella can persist for weeks in soil and dust19. So, it can live for 40 days in dry soil and 60 days in moist soils, 144 days at 20°C and 40% relative humidity, several months in drinking water at 4°C to 8°C and two and a half years at 0°C, 30 days in urine, 75 days in aborted fetuses, more than 200 days in uterine secretions, and several years in frozen tissues or culture media. In the presence of rich organic matter, Brucella resistance to various environmental conditions improved, and disease propagation via streams was rare and ineffective over short distances 20. It is an increasing genus of significant zoonotic diseases, according to Damiano et al.,21, with at least ten genetically near species occupying a variety of niches from soil to animals, cattle, and humans. It is a member of the Proteobacteria Phylum, Class (Alphaproteobacteria) Phylum, Order (Rhizobiales) Phylum, Family (Brucellaceae), Genus Brucella. B. abortus, B. melitensis, B. suis, B. canis, B. ovis, B. neotomae, B. cetaceae, B. microti, and B.pinipedia are the nine species that infect humans and animals22. It is a Gram-negative, non-motile coccobacilli. It belongs to alpha-Proteobacteria, which include in addition to Brucella other members such as Agrobacterium, Rickettsia, Rhodobacterium, and Rhizobium. However, recently atypical motile Brucella isolates were isolated from diseased frogs23. B. melitensis is the most common cause of human disease. Sheep, goat and camel are the main sources of infection24. Brucella species are among those pathogenic bacteria which have propensity to adapt to new host and they can either be naturally transmitted to their primary hosts by direct or indirect contact or sometimes inadvertently to other susceptible hosts17. Mixed farming of cows, buffaloes, sheep and goats has increased the risk of brucellosis where small ruminants act as primary hosts for B. melitensis and cattle as spillover host25. Atafar et al.,26 showed that A large amount of chemicals is annually applied at the agricultural soils as fertilizers and pesticides. Such applications may result in the increase of heavy metals particularly Cd, Pb, and As, the samples measured their heavy metal concentration, soil texture, pH, electrical conductivity, cationic exchange capacity, organic matter, and carbonate contents. The result indicated that Cd, Pb and As concentrations were increased in the cultivated soils due to fertilizer application. Brucellae are stealth microbes which prefer induction of chronic rather than acute infections27. It is a robust pathogen, with multiple routes of infection and It can resist inside and outside the mammalian hosts for a long time even under unfavorable conditions. It persists in the food up to 15 months even under unfavorable conditions as acidity and temperature between 11 and 14 °C or for 2–3 days under 37 °C. Brucella may also survive in aborted infected feti and contaminated manure for more than 2 months in winter or few hours if exposed directly to sunlight28.

2. Material and Methods

Study Area

The study area included sites of different villages for the fourth season from animal's soils from middle,south and north of Babylon province, Iraq. Theses samples included 10 sites in Winter, 32 sites in Spring,9 sites in Summer and 12 sites in Autumn, 2021.

Samples Collection (The Soil Samples)

The soils samples collected from animals' soils for the fourth season from winter in February to Autumn in November taken the samples from the upper layer for bacteriological study and from the deepest layer (25-30 cm in depth) of the soil for environmental study, dried by air and sieved (to ~ 2 mm particle

size) during the collecting the samples measured air temperature and soil temperature.

Physical and Chemical Parameters

Temperature Air and soil temperature were measured by a simple thermometer (0 ^{0}C – 100 ^{0}C), Gallen Kamp/England in the field directly.

Total Dissolved Solid (TDS) \ Total dissolved solid (TDS) was measured by multi-parameters, PCSTESTR35, Oakton - U.S.A in the field directly and expressed the units with (mg/L).

TOC (Total Organic Carbonb)

1-Weight (1gm) air \ dry soils into a (500ml) beaker.

2-Add (10ml) 1.0 N-potassium dichromate solution by pippet, then add (20ml) concentrated sulfuric acid by dispenser rotate the beaker for mix the suspension. leave it to settle down for (30 min).

3-Add (200ml) deionized water, then add (10ml) concentrated phosphoric acid,leave the mixer to cool.

4-Add (10-15) drop diphenylamine sulphate asa an indicator.

5-Titration with (ferrous sulfate solution) until the color change from (blue –violet to bright green) [29,30]

The percentage organic matter in soils

X=V sample \Vblank

Y=1- X

%O.M (w\w) = (Y*0.67*10.5) \Wt

Heavy Metals

Soils samples for heavy metals determination were digested according to the procedure described Sharidah,[31]. One gram of dried soil samples were digested with (10ml) di-acid mixture (9ml HNO_3: 4ml $HCLO_4$), the mixture was boiling gently in sandy bath on hot plate until release fumes, after cooling and filtering through Whatman No.42 filter paper and <0.45µm Millipore filter paper and transferred quantitatively to 25 volumetric flask by adding distilled water and the concentration of (Cu,Fe,Cd,Pb) were then determined with by Flame Atomic absorption - spectrophotometer (Type Aa 7000),Shimdzu /Japan was used to determine the concentrations (mg/L) of the elements.

A*B

M. Con= ———————

W

Microbiological Study:

Preparation of Culture Media:

Culture media used in this study were prepared according to the manufacturer's instructions:

Brucella Agar Base

It was prepared according to Himedia manufacturing company (India) to Isolation and detection of Brucella spp:

Suspend (21.55) gm in (500) ml D.W, heating to boiling to dissolve the medium completely. Sterilize by autoclaving at (15C^0) lbs pressure (121C^0) for 15 minutes.

Cool to (45-50 C^0) and aseptically add sterile (5%) v\v inactivated horse serum (RM 1239, inactivated by heating at (56C^0) for (30 minutes) and then add the anibiotics (Polymxin B sulphate, Bacitracin, Vancomycin, Nystatin, Nalidixic acid, Cycloheximide) as supplement.

Brucella isolation from soil

After samples collection from sites taken (1gm) from soil's sample and put in plain tube completed the size to (5ml) by normal saline then mixed by vortex and leave to settle after that incubation for (15) minute in Incubator, Two hundred (200 microliter) taken from mixing by micropipette and published in petridishs where all samples subcultured in selective Brucella agar medium with Cycloheximide (50.0mg), Vancomycin (10.0mg), polymyxin B (2,500 IU), Bacitracin (12,500 IU), Nystatin (50,000 IU), and Nalidixic acid (2.5mg) and 5% of inactivated horse serum. Bacterial cultures were incubated for 14 days at 37 ° C and 10% carbon dioxide until appearance of growth. After this step, the isolated bacteria were identified by classical typing methods 32,33,34.

Molecular Study

Molecular diagnostic methods are also currently being used for the detection of Brucella spp. in various samples 35.

Bacterial DNA Extraction:

Genomic DNA extracted from bacterial isolates cultured from the soil according to the manufacturer's protocol FavorPrep™ Blood/ Cultured Cells Genomic DNA Extraction Mini Kit.

Conventional PCR

PCR amplification was done using conventional thermal cycle as follows: Template DNA (5 µl) was added into PCR master mix tubes. Forward and reverse primers (about 10 picomols/µl) were added into PCR master mix tubes. Distilled water was added to PCR Premix tubes to a total volume of 25 µl, PCR photo is mentioned in figure (1).

The primer sequence, PCR product size and thermal cycling conditions mentioned in (1), (2) and (3).

Table (1): The sequence of primers that used in this study

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
16s RNA	F	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250 Srinivasan et al., (2015) [36]
	R	5'- GGTTACCTTGTTACGACTT- 3'	49.4	42.1	

Table (2): Reaction Components and volume for PCR from the soil and blood isolated bacteria	
Component	Final volume (25 µl)
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5 µl
Nuclease-free Water	16.5 µl

Table (3) The optimum conditions (Amplification condition) of detection the soil's isolated bacteria (Stages and Temperature of PCR for gene 16sRNA)				
		Temperature	Time	cycle
Stage 1	Initial Denaturation	95°C	5 sec	1
Stage 2	Denaturation2	95°C	45 sec	35
	annealing	56°C	45 sec	
	Extension	72°C	1 sec	
Stage 3	Final Extension	72°C	5 sec	1

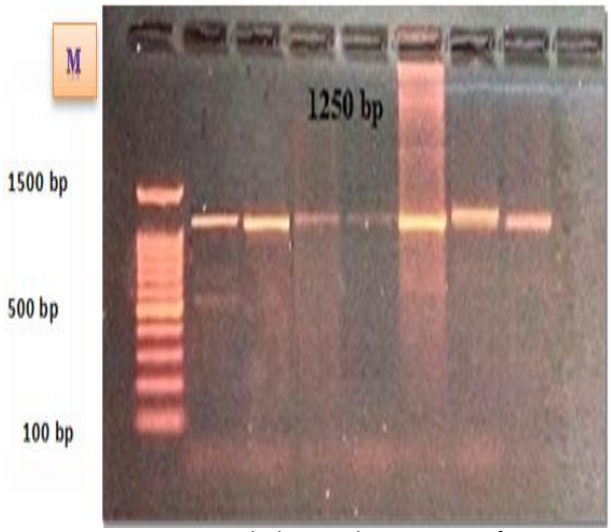

Figure (1): Agarose gel electrophoresis (2%) for 16sRNA primer-bacteria isolated from soil (1250 bp) Primer Ta at (57` C), (at 65Amp, 70 volts, 60min.). Visualized under U.V light after staining with Ethidium bromide stain, Lane M 100 bp DNA Ladder.

DNA sequencing (16S rRNA gene sequence analysis)

DNA sequencing method was performed for study of genetic changes of 16SrRNA gene in some local *Brucella* species isolates by compared with NCBI-GenBank *Brucella* species. The sequencing of the 16SrRNA gene were done after assurance in presence amplification of PCR products for required volume, The purified PCR products were sent to company (Macrogen) in Korea for performed Sanger sequence, after getting on nitrogenous bases sequence for 16SrRNA gene amplified products of *Brucella* isolates, this sequence analyzed by NCBI-blast for purpose compared homology or diversity degree to local Brucella isolates with the world isolates recorded in NCBI-GenBank. Where founded after the sequence results analysis (37) of local Brucella isolates found 28 new isolate for 11 Brucella species that include (***Brucella pseudogrignonensis, Brucella rhizosphaerae, Brucella oryzae, Brucella intermedia, Brucella anthropic, Brucella ovis, Brucella inopinata, Brucella melitensis, Brucella lupini, Brucella pituitosa, Brucella thiophenivorans***) for soil deposited in Genbank considering the percent identity. (37) in soil were used for partial sequence alignment (except one with complete sequence).

Occurance types of substituations mutations (Transvertion\Trasition) for soil isolates of (different villages from Babylon province middle, north and south) for 11 species of *Brucella* by compared local one strain with strains to one states of the world states. This evidence for presence genetic diversity with the isolates, these sequences recorded and published in the Gen Bank database taken as a reference to identify the polymorphisms.

The results and discussion

Environmental parameters have been recorded during this study for four season, Bacteria percentage (%) have the lowest rate in the Winter season while the higher rate in the Summer season.

Table (4): The relationship between the fourth seasons and the physical, chemical parameters and the percentage of bacteria (Mean ± S.D). p≤0.05				
SeasonParameters	Winter	Spring	Summer	Autumn
Bacteria percentage (%)	60±0.5[a]	78.13±9.5[b]	100±11.4[c]	91.67±6.7[c]
Air Temp.	27.40±4.9 [a]	30.69±4.8 [ab]	42.11±2.4[c]	32.83±1.8[b]
Soil temp.	21.85±2.8 [a]	26.17±3.4 [ab]	37.11±3.1 [c]	23.01±7.3 [a]
pH	7.62±0.3[a]	7.56±0.4[a]	7.81±0.2 [b]	7.51±0.3[a]
EC (µs/cm)	3957.0±14.5 [a]	3768.34±22.6 [a]	4958.89±27.4 [b]	5123.33±18.1 [b]
TDS (mg/L)	2812.0±10.5 [b]	2645.94±15.8 [a]	3548.89±21.06 [c]	3593.33±12.7[c]
Salinity (‰)	2.53±0.9 [a]	2.47±0.3 [a]	3.17±0.7 [a]	3.28±1.1[a]
TOC (%)	42.44±11.3 [c]	35.68±17.1 [b]	34.57±6.9 [b]	27.63±5.2[a]

Table (5): The relationship between the Seasons with the Bacterial Presence and The Concentrations of Heavy Metals (Mean±S.D). p≤0.05

Season Parameters	Winter	Spring	Summer	Autumn
Bacteria percentage (%)	60±0.5a	78.13±9.5 b	100±11.4 c	91.67±6.7 c
Cu (mg\kg)	12.51±6.6 b	5.36±1.3 a	4.11±5.6 a	5.95±1.2 a
Fe(mg\kg)	412.29±20.8 a	304.08±23.2 a	314.95±18.8 a	392.44±21.8 a
Cd(mg\kg)	1.88±0.3b	0.52±0.02 a	1.17±0.2 b	1.77±0.1 b
Pb(mg\kg)	647.99±174.3 a	726.97±141.9 b	731.77±33.1 b	722.32±32.1b

The results show that there is relationship between the *Brucella* percentage with environmental parameters and heavy metals according to seasons. Our the results proved existence relation between the presence of bacteria and the physical, chemical properties. In this study noted that proportion of bacteria presence in summer is higher that is in relation with environmental parameters include (Air and Soil temperature, pH, EC, TDS and Salinity) is high, except TOC is low while proportion of bacteria presence in winter is lower, also relate with environmental parameters include (Air and Soil temperature, pH, EC, TDS and Salinity), these the parameters (the physical and chemical) is low except TOC is high. This is indicate that variation of seasons affect to *Brucella* ratio in soil as in table (4). While the results confirmed existence relation between the presence of bacteria and the heavy metals, as mentioned in table (5). In the current study, it was observed that the heavy metals affect the percentage of Brucella percent in soil, The high amounts of Cu,Fe,Cd are in winter, when the percentage of Brucella is low, except for lead (Pb), which is low in the presence of little *Brucella*. while low amounts of copper,iron, cadmium be in the proportion of the presence of high Brucella, except for lead (Pb), which is high in the presence high *brucella*.

These the results correspond with Ahmed et al.,[37] that Animals are infected by Brucella species, which also have a zoonotic effect. Using a metagenomics approach, risk variables related with the presence of Brucella species in soil were studied., soil samples (n= 1280) were collected from 256 villages (5 samples/village) across Punjab's nine districts: Lahore, Physical risk factors such as distance from water irrigation source, distance from animal market, and animal density per village were all found to be significantly associated with the prevalence of soil borne *Brucella* species, whereas other risk factors such as distance from main road and number of houses per village were found to be less so. Chemical risk factors are linked. The rate of Brucella was higher in summer in agreement with Salari *et al.*,[38] showed that prevalence brucellosis was highest in summer (39.5%) and more common males than among females. Prevalence was highest among those aged 10-19 years [27.7%]. Most patients had a history of infected cheese, milk and milk product consumption [98%). Also, our results was coincide with Liu *et al.*,[39] The incidence was high in summer and autumn, and the peak was in May, Six virulence genes were found in two isolated strains, suggesting that the *Brucella melitensis* strains in this study had strong virulence. The soil is the largest carbon reservoir in the terrestrial ecosystem, and thus small changes in soil carbon might greatly affect the carbon balance in the ecosystem[40]. In addition to vegetation and soil microbes, there are numerous reports showing close relationships between environmental parameters and SOC content, stocks, or turnover[41], relatively fewer studies have focused on the relationships between these abiotic factors and SOM composition, air temperatures, land uses. However, there are several studies showing that multiple factors interact with SOM. Wang *et al.*,[42] reported that temperature, rainfall, soil order, landscape, and land-use could explain the variability of SOM composition after conducting SOM analysis across a wide range of grasslands in New Zealand. Vancampenhout *et al.*,[43] also examined several soil variables controlling SOM composition, including dominant vegetation. Thus, soil environmental variables should be considered to understand the multidimensional nature of SOM composition. The niche of a species in a particular ecosystem helps set up the features of its environment that are crucial for its survival and the interrelationship of a species and its relational position in a particular ecosystem, including the relationship of the species with the components of the ecosystem itself and the niche may be influenced by all the factors included in its ecosystem and the niche of a species in a particular ecosystem helps set up the features of its ecosystem that are crucial for its survival[5]. Air temperature is the key microclimatic parameter, which is affected by other physical factors (air flow, air humidity). A comfortable temperature range depends on animal species, age and total metabolic level[44]. Incidence rates in Iraq and Egypt, for example, have been reported to vary by 4–5 times depending on the area. This emphasizes the impact of regional, environmental, socioeconomic, and lifestyle factors on the disease's occurrence[45]. The ecology of brucellosis is changing dramatically as a result of changes in lifestyle and the environment, the ecology of brucellosis is changing dramatically as a result of changes in lifestyle and the environment[46], resulting in an unequal distribution of the illness around the world. In comparison to Europe and North America, as well as other industrialized regions of the world, the Middle East, particularly Syria, Iraq,

Egypt, Turkey, and Iran, bears the brunt of the disease's burden[47]. Soils with different organic matter contents can be obtained using samples from a variety of sites or from a single site where different organic matter contents have developed as a result of differences in crop management practices, or by the use of soil samples from a single site with different organic matter contents[48]. To better understand the nutrient dynamics in tree species, Babur and Dindaroglu,[49] looked at seasonal changes in microbial activity and microbial biomass, as well as the consequences of seasonal changes (mainly temperature and water content). Microbial population and activity levels varied dramatically with the seasons and followed a predictable pattern (summer > autumn > spring > winter). The availability and release of nutrients in an ecosystem are influenced by soil organic matter decomposition by soil microbes. In soil aggregation, soil porosity, moisture content, and aeration, improving these two critical soil components can help with climate change adaptation and mitigation. According to Pakzad *et al.*,[50], there are significant disparities in the spatial distribution of this disease in Iran. The cities of the west and north-west of the country have high brucellosis incidence rates, and the greater occurrence of the disease in the summer has resulted in an increase in health costs for people of these locations. As a result, it's critical to precisely define the disease's environmental, economic, and social elements in order to determine where the biggest risk locations are and when they're most dangerous. While the results by Aune *et al.*,[51] during experiments established that *Brucella* bacteria can persist on fetal tissues and soil or vegetation for 21–81 days depending on month, temperature, and exposure to sunlight. Organic matter inputs in the form of manure or straw, either alone or in conjunction with chemical fertilizers, appear to be more successful than chemical fertilizer alone in preserving or recovering organic matter in soil, according to Wu *et al.*,[52]. Heavy metals provide a significant environmental danger since they are not biodegradable and so endure, resulting in ongoing increases in concentrations[53]. Soil microorganisms' activity and community structure can successfully function as markers of soil environmental quality[54]. Soil microorganisms' activity and diversity are highly sensitive to inorganic and organic contaminants[55].

Table (6): The relationship between genotype and phenotype parameters for ecological and heavy metals for (*B. melitensis* and *B. pseudogrignonensis*), Mean ±S.E. p≤0.05

	B. melitensis gene polymorphisms						P=value
	T\C	C\A	C\G	A\G	C\G	T\G	
Air Temperature	40.50± 0.50	36.66± 1.76	30.50± 0.50	29.50± 0.50	34.50± 0.50	33.50± 0.50	0.003*
Soil Temperature	34.70± 0.30	28.46± 4.06	25.25± 0.25	25.75± 0.25	24.70± 0.30	25.70± 0.25	0.19
pH	8.70± 0.10	7.43± 0.26	7.55± 0.05	7.60± 0.10	6.95± 0.05	7.45± 0.05	0.004*
EC	9790.50± 0.50	4020.00± 99.00	3505.00± 5.00	3495.00± 5.00	3852.00± 2.50	2457.50± 2.50	<0.001*
TDS	7270.50± 0.50	2710.00± 68.00	2440.50± 0.50	2439.50± 0.50	3930.50± 0.50	1730.00± 0.50	0.001*
Salinity	6.25± 0.05	2.57± 0.63	2.25± 0.05	2.30± 0.01	3.75± 0.05	1.55± 0.05	<0.001*
TOC	100.50± 0.50	31.19± 4.98	18.35± 0.35	17.85± 0.85	40.50± 0.50	11.50± 0.50	<0.001*
Cu	1.85± 0.05	9.35± 0.05	1.25± 0.05	1.15± 0.05	4.40± 0.10	0.45± 0.05	0.30
Fe	387.50± 0.50	306.33± 12.00	227.50± 0.50	226.50± 0.50	321.50± 0.51	162.50± 0.50	0.47
Cd	1.25± 0.05	1.53± 0.19	1.35± 0.05	1.25± 0.06	1.75± 0.05	1.95± 0.05	0.035*
Pb	693.50± 0.50	738.15± 6.57	694.00± 5.00	688.00± 0.5	746.00± 0.50	667.00± 0.50	<0.001*

		B. pseudogrignonensis gene polymorphisms			P= value			
	A\C	A\G	G\C	T\G	G\A	T\A	C\A	
Air Temperature	27.00± 2.56	24.00± 4.00	29.33± 4.66	27.00± 7.00	33.50± 0.50	34.50± 0.50	32.50± 0.50	0.52
Soil Temperature	21.45± 1.06	18.85± 1.50	23.30± 1.10	23.85± 1.60	25.75± 0.25	24.75± 0.75	23.65± 0.35	0.06
pH	7.63± 0.13	7.40± 0.10	7.73± 0.24	7.50± 0.10	7.55± 0.05	7.45± 0.05	7.56± 0.05	0.83
EC	5256.66± 281.48	4215.00± 745.33	4993.33± 997.03	5770.00± 770.00	6256.00± 5.00	6255.00± 5.00	6356.00± 5.00	0.34
TDS	3663.33± 87.00	3010.33± 54.00	3483.00± 69.00	3420.00± 12.20	3458.00± 2.00	4457.00± 2.50	3815.00± 5.00	0.58
Salinity	3.03± 0.33	2.69± 0.47	3.19± 0.63	3.09± 1.09	4.25± 0.25	4.20± 0.20	3.70± 0.30	0.41
TOC	47.96± 4.07	48.21± 1.75	51.27± 7.62	47.55± 11.00	35.25± 0.25	35.40± 0.35	28.50± 0.50	0.15
Cu	10.95± 3.23	20.37± 1.96	6.28± 2.33	7.43± 3.21	2.87± 0.02	2.78± 0.08	00.00± 00.00	0.05*
Fe	496.34± 52.09	389.41± 31.33	534.88± 55.04	518.69± 29.00	514.00± 1.00	513.00± 0.50	437.00± 0.50	0.79
Cd	0.94± 0.30	2.03± 0.34	0.22± 0.05	0.23± 0.08	1.65± 0.005	1.64± 0.005	1.85± 0.50	0.028*
Pb	683.64± 11.95	663.01± 8.84	692.24± 21.70	678.48± 28.73	750.00± 1.00	571.50± 0.50	734.00± 1.00	0.019*

These tables show that ***B. melitensis*** and ***B. pseudogrignonensis*** occurred substituation mutation and noted presence significant differences ($p<0.05$) between genotype and phenotype that include physical and chemical parameters (Air and soil temperature, pH, EC, TDS, Salinity, TOC) and heavy metals (Cu,Fe,Cd,Pb) according to statistical analysis, this indicate to presence genetic variation in them, This indicate exist The polymorphism of these bacteria due to environmental indicators and heavy elements caused the bacteria to change, this change in the structure of DNA and change in the nucleotide bases so that it adapts to the external environmental conditions and can resist and survive. Also, its resistance to the toxic heavy elements present in the soil has been transformed into new strains in this soil to be able to survive, this genetic change in bacteria causes the moral differences between them. *B. melitensis* founded in summer and autumn seasons while *B. pseudogrignonensis* existed in Winter, Spring and Autumn seasons.

Brucella is a robust pathogen, with multiple routes of infection. It can resist inside and outside the mammalian hosts for a long time even under unfavorable conditions. It persists in the food up to 15 months even under unfavorable conditions as acidity and temperature between 11 and 14 °C. or for 2–3 days under 37 °C. Brucella may also survive in aborted infected feti and contaminated manure for more than 2 months in winter or few hours if exposed directly to sunlight28. It was considered to be a facultative intracellular pathogen in most references; however, they were re-designated as facultative extracellular intracellular pathogens due to their evolutionary relationship to other alpha-Proteobacteria56. Due to the high genomic homology among the typical Brucella species, it was supposed in the 1980 s that Brucella is a monospecific genus (Brucella melitensis) which has 6 biovars distinguished according to their host prevalence, the different Brucella species were renamed, However this classification did not survive the new data delivered by molecular biological genotyping tools 57. Brucella was always considered to be an animal pathogen with a high zoonotic impact and infected humans are the dead end of the disease. However, it was proven recently that man to man infection is possible. This may be related to the continuous improvement in the diagnostic and epidemiological tools, or to the continuous adaptation of the organism to their hosts23. Brucella species that have recently been identified have a lot of genetic plasticity. Many of these isolates are mobile, quick growth that can survive in the soil, are more resistant to high acidity and unfavorable climatic conditions and have a high ability for adaptation to new non-mammal hosts like amphibians. They also have a high metabolic activity. They may quickly adapt to their surroundings in order to expand their host range,The genetic diversity of newly discovered Brucella species is much greater than that seen among thousands of isolates of classical Brucella species discovered throughout the twentieth century. A close genetic link exists between these atypical Brucella species and soil microorganisms. This prevents them from acquiring new genetic features from the environment23. It was long thought to be an animal pathogen with a strong zoonotic potential, with infected people serving as the disease's final destination. Man-to-man infection, on the other hand, has recently been proven. This could be due to advancements in diagnostic and epidemiological methods, as well as the organism's ongoing adaptability to its hosts28. In Taiwan, this is the first case of O. pseudogrignonense infection and whole genome sequencing. O. pseudogrignonense is a rod-shaped bacterium that is gram-negative, non-motile, non-spore-forming, and oxidase-positive58. It's a type of environmental organism that can be found in both water and soil59.In 2016, whole genome sequencing of O. pseudogrignonense from Malaysian tropical soil was reported. Clinical cases were reported from a 28-year-old patient's blood and a newborn's ear in Sweden in 1992 and Norway in 2000, respectively58. A human case report published recently in Korea described bacteraemia in a man receiving extracorporeal membrane oxygenation60. Cadmium is emitted into the atmosphere as a result of natural or human-made activity, and it can affect both animals and humans in diverse ways. It has no properties that are beneficial to plant development or metabolic activities61. Lead is a non-biodegradable metal that is found in relatively small amounts in nature. Because of human activities such as manufacturing, mining, and burning fossil fuels, atmospheric lead levels are steadily rising. When exposed to levels higher than the ideal, lead is hazardous to the human body. Children are more susceptible to lead poisoning, and the degree of poisoning rises when they come into touch with dust contaminated with environmental lead62. The potential for heavy metals to migrate from polluted land and water into the food chain makes bioaccumulation of heavy metals in vegetables a health risk63.Heavy metal pollution, absorption and bioaccumulation in food crops, poses significant environmental and health issues, particularly in developing nations. Soil type, plant genotype, and their interactions all have an impact on heavy metal concentrations64. Mineral fertilizers, in compared to organic manure, contain higher concentrations of heavy metals; as a result, the use of mineral fertilizers results in higher levels of heavy metal contamination in soil65. According to the findings of Atafar et al.,66, fertilizer application enhanced Cd, Pb, and as concentrations in agricultural soils. Despite the fact that statistical analysis shows that these heavy metals increased significantly (Pvalue=0.05), lead and arsenic concentrations increased dramatically in comparison to Cd concentrations and a decrease in pH, increasing heavy metal availability and aggravating the problem of deteriorating food

quality, metal leaching, and impacts on soil organisms67. Heavy metal concentrations in soil are determined by the geological properties of the soil. Normal farming practices, on the other hand, tend to accumulate these elements. The use of liquid and soil manure (or their derivatives, compost, or sludge) or inorganic fertilizers causes these metals to accumulate in the soil. These practices are key heavy metal sources68.

Table (7): The relationship between genotype and phenotype parameters for ecological and heavy metals for B.lupini and B.inopinata, Mean ±S.E. p≤0.05

	B.lupini gene polymorphisms		P=Value	
	A\G	T\A	G\T	
Air Temperature	43.50± 1.25	44.66± 0.66	45.50± 0.05	0.49
Soil Temperature	37.17± 1.84	37.36± 2.54	42.50± 0.50	0.28
pH	7.75± 0.14	7.63± 0.12	7.76± 0.50	0.79
EC	5125.00± 20.00	5996.00± 24.09	6001.00± 1.00	0.89
TDS	3642.00± 40.00	4296.00± 30.00	4230.00± 50.00	0.88
Salinity	3.27± 0.95	3.83± 1.09	3.58± 0.05	0.9
TOC	23.09± 9.08	29.28± 9.00	9.95± 0.05	0.52
Cu	2.61± 0.29	2.87± 0.17	2.55± 0.05	0.67
Fe	207.86± 11.00	141.32± 10.00	6.35± 0.055	0.55
Cd	1.28± 0.06	1.31± 0.07	1.45± 0.052	0.32
Pb	749.20± 14.26	742.57± 17.86	711.50± 0.50	0.32

While ***B. lupini and B. inopinata***, don't have significant differences (p>0.05) between genotype and phenotype, this indicate that genetic variation is present but don't affect on bacteria composition. *B. lupine* founded in summer season while *B. inopinata* existed in summer and spring. *Brucella lupini* is a root-nodulating bacterium that is non-rhizobial. Its name comes from the fact that it nodulates Lupinus albus. The type of strain is LUP21T (LMG 20667T)[69]. Wherease *Brucella inopinata*, on the other hand, is a Gram-negative, nonmotile, nonspore-forming coccoid bacteria that was originally isolated from a breast implant infection site. BO1T (=BCCN 09-01T =CPAM 6436T) is its type strain[70]. It's a possible source of brucellosis. Thacker *et al.*,[71] discovered that a locally isolated gram-negative *Brucella* sp. strain, identified using biochemical techniques and 16SrRNA analysis, was found to be virulent. Multiple heavy metal (Ni, Zn, Hg, Pb, Co) tolerance and antibiotic resistance were found in the strain. When heavy metals impacted soil microbial communities, autochthonous lead- and cadmium-resistant isolates were discovered, which had a negative influence on biomass, metabolic activity, and diversity. The microbial community in several metal-stressed soils comprised of two groups, one resistant to lead and the other vulnerable to it. A lead-resistant isolate was also isolated from a control soil that had never been exposed to lead, implying widespread lead resistance. These findings demonstrated that heavy metals have an impact on the gut flora[72]. Wild animals serve as reservoirs for causative agents that can lie dormant for lengthy periods of time until they are activated. The presence of agents in uncommon hosts can occasionally raise the risk of RNA replication mistakes, which can result in mutations. This can result in the emergence of novel strains or species that are more virulent and/or antibiotic resistant. Increased transmission rates in sensitive groups could be one of the consequences [73]. Disease reservoirs in the environment are still being recognized as important factors in disease outbreaks, pathogen persistence, and ongoing transmission in humans[74], Animal manures include a variety of zoonotic infections, according to Dungan,[75], which are suspected of being carried off-site as aerosols from confined feeding operations. For bacterial identification and phylogenetic classification, DNA extracts from aerosol samples were produced, and a portion of the 16S ribosomal RNA gene was sequenced. Although the findings imply that aerosolized bacteria are diverse, since 2007, novel Brucella strains have been identified having phenotypic traits that are more similar to those of strains belonging to the genus Ochrobactrum than to those of classic *Brucella* species[76]. Although they are genetically more distant from the traditional Brucella species, which comprise a homogenous ancestral to the classic Brucella species, Molecular analysis indicated that these strains belong to the genus Brucella. *B. inopinata*, on the other hand, is more distant from classic *Brucella* species, while it is closer to them than Ochrobactrum species[77]. Within *B. inopinata*, only the type of strain BO1 has been classified. Other *Brucella* strains, genetically distinct from the traditional species, have been described, including isolates from wild rats in Australia and frogs from Africa[78]. As *B. inopinata*, strain

BO2 and strains from wild Australian rats are among the genetically distant strains from the traditional Brucella species that have recently been discovered[79]. BO1 and *Brucella* species were the most vulnerable to *Brucella* infection[80], with mice being the least susceptible. Amphibian isolates show a degree of horizontal gene transfer, with the insertion of genomic areas with sequence identity to soil dwelling or facultatively pathogenic Alphaproteobacteria, most notably from the genus Ochrobactrum[23]. Any trace elements, necessary or non-essential, present in excess of safe amounts can cause physiological or morphological abnormalities, as well as genetic alterations, such as slowing or stopping growth or producing mutations[81]. Based on 16S rRNA sequences, one group's sequence is identical to the *B. inopinata* consensus sequence, whereas the other group's sequence has an Ochrobactrum-derived 44-nucleotide insertion[23].

Trujillo et al.,82 demonstrated that nodulation of legumes has been thought to be the exclusive domain of a group of microbes known as rhizobia and belonging to the Proteobacteria for more than a century. Lupinus plants were re-infected with the strains. Because the strains recovered in investigation have molecular characteristics belong to a new species of Ochrobactrum, was named Ochrobactrum lupini sp. Nov.

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