Article

Not peer-reviewed version

The Toxic Effect of Heavy Metal on *B.intermedia* and *B. rhizospherae* and Its Relation with the Seasonal Environmental Factors

Ayad Almamoori *, Wasan Mohammed Abdulzahra , Jason Rao

Posted Date: 29 December 2023

doi: 10.20944/preprints202312.2292.v1

Keywords: Brucella sp.; heavy metals; Environmental factors; Toxic effect

Article

The Toxic Effect of Heavy Metal on *B.intermedia* and *B. rhizospherae* and Its Relation with the Seasonal Environmental Factors

Wasan Mohammed Abdulzahra [1,*], Ayad M. J. Al-mamoori [2] and Jason Rao [3,*]

[1] University of Babylon, Faculty of Basic Education; ayadmj77@gmail.com
[2] Biology Dept., College of Science, University of Babylon
[3] Cornell University U.S.A

Abstract: This investigation aimed to knowledge of the presence of *Brucella* spp. in the samples from Babylon province and the relationship with environmental factors. The seasonal collection of animal soil samples for various villages in the middle, south, and north of Babylon province, Hilla City, Iraq, with physicochemical parameters (air and soil temperature, pH, EC, TDS, salinity, TOC), as well as heavy metals (Fe, Cu, Cd, and Pb). The results showed the presence of *Brucella* species in some of soil samples and its percentage varies according to the four seasons, where the highest percentage of *Brucella* spp. was in summer season and the lowest in winter season and these percentages were related to seasonal environmental indicators. After sending the positive bacterial samples isolated from the soil for sequencing analysis, it was found that the bacterial samples isolated from the soil have new strains recorded in Genbank as following: five strains for *Brucella intermedia* and one strain for *Brucella rhizosphaerae,* Also, phylogenetic analysis was for the drawing of phylogenetic tree and the determination of phylogenetic relationship for Knowledge the degree of convergence between species and their source. Through our research, we concluded that proportion of *Brucella* spp. presence in summer was high and also environmental factors .This indicated that differences of seasons affect to *Brucella* ratio in soil. The findings of sequencing for the bacterial samples from soil demonstrated that *B. intermedia* was found in both the spring and fall seasons while *B.rhizosphaerae* found in the spring season , These types of *Brucella* had genetic variations.

Keywords: Brucella sp.; heavy metals; Environmental factors; Toxic effect

Introduction

Brucella belongs to the Brucellaceae family, the orders Rhizobiales, Daeguia, Crabtreella, Mycoplana, Pseudochrobactrum, and Paenochrobactrum are all members of the Alpha proteobacteria class[1]. It is heat sensitive and can survive for several weeks in water. The survival of *Brucella* abortus in the environment is influenced by sunlight and temperature[2]. Bacterial development and diversity are linked to organic matter; thus, microbial numbers are greatest at the soil's surface (10 cm) and decrease with depth[3]. Identify different ecotypes and the potential for new strains, giving them insight into the environmental maintenance and transmission of emerging and re-emerging disease risks in Iraq. The presence of *Brucella* species in soil is one of the risk factors for the disease's spread to animals and humans[4]. Brucellosis is a zoonotic infection that can affect both animals and humans and it is still prevalent in the United States[5]. *Brucella* species are pathogenic bacteria that adapt to new hosts and are naturally transmitted to their primary hosts by direct or indirect contact, as well as to other vulnerable hosts unwittingly[6]. The use of cows, buffaloes, sheep, and goats in mixed farming has increased the risk of brucellosis with small ruminants acting as primary hosts and cattle acting as an overflow host for *B. melitensis*[7]. The current study investigates the association between soil risk variables (physical and chemical) and the occurrence of *Brucella* species. the epidemiological relevance of brucellosis in terms of risk of transmission to humans and cattle[8]. Seiler & Berendonk, (2012)[9] showed that the risk of metal resistance in the environment was

 doi:10.20944/preprints202312.2292.v1

assessed based on heavy metal concentrations, Analyses of the data indicate that agricultural and aquacultural practices represent major sources of soil and water contamination with moderately to highly toxic metals such as mercury (Hg), cadmium (Cd), copper (Cu), and zinc (Zn). If those metals reach the environment and accumulate to critical concentrations, they can trigger co-selection of antibiotic resistance. Li *et al*.,(2013)[10] found that incidence of brucellosis were strongly associated with lower temperatures and less sunshine in the winter and spring, climatic factors likely influence the ecology of brucellosis both directly and indirectly by affecting several parameters, including the growth and reproduction dynamics of domestic animals, interactions between sheep, goats and humans, pathogen replication, and population immunity[11]. Ahmed *et al*., (2017)[12] discovered DNA of *Brucella* species in soil samples (n=1280) from nine districts in Punjab.

Material and methods

The Soil Samples Collection

The animals soil samples collected seasonally from February to November, 2021 were taken the samples from the upper layer for bacteriological study and from the deepest layer (25-30 cm in depth) for environmental study, dried by air and sieved (to ~ 2 mm particle size) during the collecting the samples measured air temperature and soil temperature and determination the sites by GPS.

Total Organic Carbon is the wet oxidation technique was utilized exothermic heating and oxidation of organic carbon of the sample with potassium dichromate by the titration[13], **Heavy Metals** include soils samples for heavy metals determination were digested according to the procedure described Sharidah (1999)[14]. One gram of dried soil samples were digested and determined with by Flame Atomic absorption - spectrophotometer (Type Aa 7000),Shimdzu /Japan was used to determine the concentrations (mg/L) of the elements[15].

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(21.55gm) in (500ml) D.W,heating to boiling to dissolve the medium completely. sterilize by autoclaving at (15^{0}C) Ibs pressure (121^{0}C) for 15 minutes ,Cool to (45-50^{0}C) and aseptically add sterile (5%) v\v inactivated horse serum (RM 1239, inactivated by heating at (56^{0}C) for (30 minutes) and then add the antibiotics (Polymxin B sulphate ,Vancomycin, Bacitracin, Nystatin, Nalidixic acid, Cycloheximide) as supplement .

Brain Heart Infusion Broth (BHI)

This media were prepared according to the manufacturer's instruction. It was used for preservation of bacterial isolates as stock for long time[16] .

***Brucella* isolation from soil samples**

After samples collection in sterile bags 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 µl) were taken from mixing by micropipette and published in petridishs where all samples subcultured in selective Brucella agar base media with Polymyxin B (2,500IU), Vancomycin(10.0mg), Bacitracin (12,500IU), Cycloheximide(50.0mg), Nystatin(50,000 IU) and Nalidixic acid (2.5mg) and then5% of inactivated horse serum. Bacterial cultures were incubated for 14 days at (37°C) and 10% carbon dioxide until appearance of growth[17,18,19] .

Molecular diagnostic methods

Methods are also currently being used for the detection of *Brucella* spp. in various samples[20].

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.

 doi:10.20944/preprints202312.2292.v1

Conventional PCR for Bacteria Extracted from Soil

Conventional PCR were used to amplify the target bacterial DNA using specific primer pairs. It includes three consecutive steps that repeated for specific number of cycles to get PCR product which can be finally visualized after agarose gel electrophoresis. The primer sequence, PCR product size and thermal cycling conditions mentioned in (table 1-1) and (table 1-2).

Table 1-1. The Sequence of Primers.

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
16s rRNA bacterial primers	27F	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250 Srinivasan *et al.*, (2015)[21]
	1392R	5'- GGTTACCTTGTTACGACTT- 3′	49.4	42.1	

Table 1-2. The Optimum Conditions for Detection the Bacterial Isolates from Soil (Stages and Temperature of PCR for *16s rRNA* gene).

		Temperature ^{0}C	Time	cycle
Stage 1	Initial Denaturation	95ºC	5 min	1
Stage 2	Denaturation	95ºC	45 sec	
	annealing	56ºC	45 sec	35
	Extension	72ºC	1 min	
Stage 3	Final Extension	72ºC	5 min	1

16. S rRNA Sequence Analysis and Phylogenetic Tree

Sequencing method was performed for study of genetic changes and phylogenetic tree draw of *16SrRNA* gene in some local *Brucella* isolates by comparing with NCBI-GenBank *Brucella* isolates. The sequencing of the *16SrRNA* gene were done after assurance in presence amplification of PCR products for required volume; These products were sent to company (Macrogen) in Korea for performing Sanger sequence , after getting on nitrogenous bases sequence for *16S rRNA* gene amplified products of *Brucella* isolates. This sequence analyzed by NCBI-blast programme for purpose compared homology or diversity degree to local *Brucella* isolates with the world isolates recorded in NCBI-GenBank. Also phylogenetic analysis for draw of phylogenetic tree and determination phylogenetic relationship used (MEGA 6) programme to compare local one strain with strains all of the world states.

Recording of Iraqi *Brucella* isolates in gene bank –NCBI

Sequences of *Brucella* isolates were isolated from animals soils in Al-Hilla city\Iraq and each sequences have variations.

Results

Soil Samples

The current study results showed that Brucella spp. was found in all seasons,

Genetic Detection

Conventional PCR for Bacterial Isolates and 16SrRNA Sequencing

After the culture for both samples from the soil on Brucella agar base media and DNA extraction from bacterial isolates and electrophoresis of DNA as in Figure (1-1) after that, identified the positive samples by conventional PCR to knowledge *Brucella* sp., as in Figure (1-2).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 29 December 2023 doi:10.20944/preprints202312.2292.v1

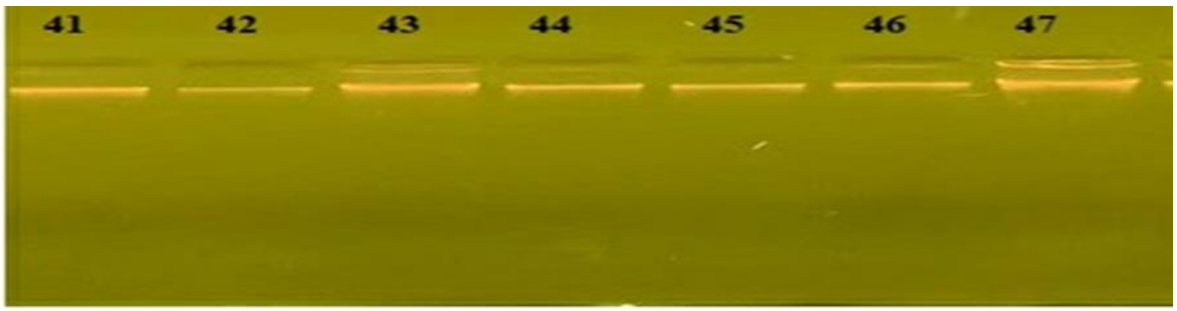

Figure (1-1). Gel electrophoresis for Extracted DNA from soil bacterial isolates, (Agarose 1%, at 70 volts, 60 min). Visualized after staining with ethidium bromide stain.

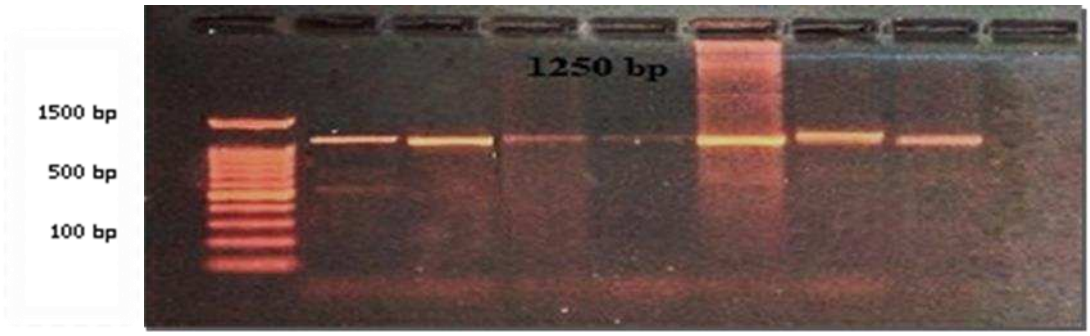

Figure (1-2). Agarose gel electrophoresis (1%) for 16s-rRNA bacterial primer-bacteria isolated from the soil samples (1250 bp), Primer Ta at (560C), at (65Amp ,70 volts, 60min). It was visualized under U.V light after staining with Eco Safe dye , Lane M 100 bp DNA Ladder.

After Sequencing analysis for both bacteria isolated from soil and blood samples found that *Brucella* isolates from soil samples for the four seasons and sented for sequencing was 6 new strains recorded in GenBank mentioned in table (1-3). These samples divided to (5) new strains for *Brucella intermedia* in Summer and in Autumn, one species of *Brucella rhizosphaerae* in Spring.

Table 1-3. Brucella isolates recorded in GenBank for Brucella spp. isolated from soil.

Isolate name	Accession number	*Brucella sp.*
AyJaWa-5	OM246513	*B.intermedia*
AyJaWa-6	OM246514	*B. intermedia*
AyJaWa-36	ON158074	*B. intermedia*
AyJaWa-38	ON158076	*B.intermedia*
AyJaWa-41	ON158079	*B.intermedia*
AyJaWa-42	ON158080	*B.rhizosphaerae*

The results of 16SrRNA sequencing revealed the presence variation for Brucella isolates from soil established (6 new strains) for two species, indicating the presence of genetic diversity among the isolates.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 29 December 2023 doi:10.20944/preprints202312.2292.v1

Table (1-4). The relationship between *B.intermedia* and environmental factors, Mean ±S.E. p≤0.05.

Parameters	*B. intermedia*	P=value
Air Temperature (⁰C)	30.238±1.282	0.38
Soil Temperature (⁰C)	24.53±1.394	0.65
pH	7.622±0.278	0.46
EC (µs/cm)	5134.73±1301.224	0.47
TDS (mg/L)	4037.7965±902.598	0.30
Salinity (‰)	3.296±0.844	0.45
TOC (%)	30.294±4.142	0.05*
Cu (mg\kg)	11.54±1.926	<0.001*
Fe (mg\kg)	513.178±57.904	0.001*
Cd (mg\kg)	1.326±0.166	0.004*
Pb (mg\kg)	746.268±16.602	0.14

This table (1-4) showed that all isolates have significant differences (p<0.05) between genotype and environmental factors that included physical and chemical parameters (air and soil temperature, pH, EC, TDS, salinity, TOC) and heavy metals (Cu, Fe, Cd ,Pb) according to statistical analysis.

Table (1-5). The relationship between *B.rhizosphaerae* and environmental factors, Mean ±S.E. p≤0.05.

Parameters	*B.rhizosphaerae*	P=value
Air Temperature (⁰C)	21.5±0.57	0.14
Soil Temperature (⁰C)	24.38±0.385	0.23
pH	7.15±0.05	0.14
EC (µs/cm)	3665±5.735	0.143
TDS (Mg\L)	2559.165±3.73	0.39
Salinity ‰	2.355±0.04	0.22
TOC %	35.63±0.065	0.13
Cu (mg\kg)	5.45±0.05	0.14
Fe (mg\kg)	584.5±0.685	0.14
Cd (mg\kg)	0.1415±0.0065	0.38
Pb (mg\kg)	596.5±0.725	0.4

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 29 December 2023 doi:10.20944/preprints202312.2292.v1

While *Brucella rhizosphaerae* mentioned in tables (1-5) do not exhibit significant differences ($p>0.05$) between genotype and environmental parameters, this suggests that genetic diversity is present but has no impact on the makeup of the bacterium. *B.rhizosphaerae* founded one in the spring season .

Phylogenetic Tree Draw

Sanger sequencing was successful for soil samples with an expected PCR product size (1250 bp) by 16S rRNA bacterial primers. The sequences were identified as belonging to *Brucella* spp. following similarity searched by blast (sequence identity of 99%). 16S rRNA sequencing method was performed for study of genetic changes and phylogenetic tree draw of *16SrRNA* gene in some local *Brucella* species isolates by compared with NCBI-GenBank *Brucella* species. The sequences were deposited in GenBank with accession numbers as mentioned in table (1-6).

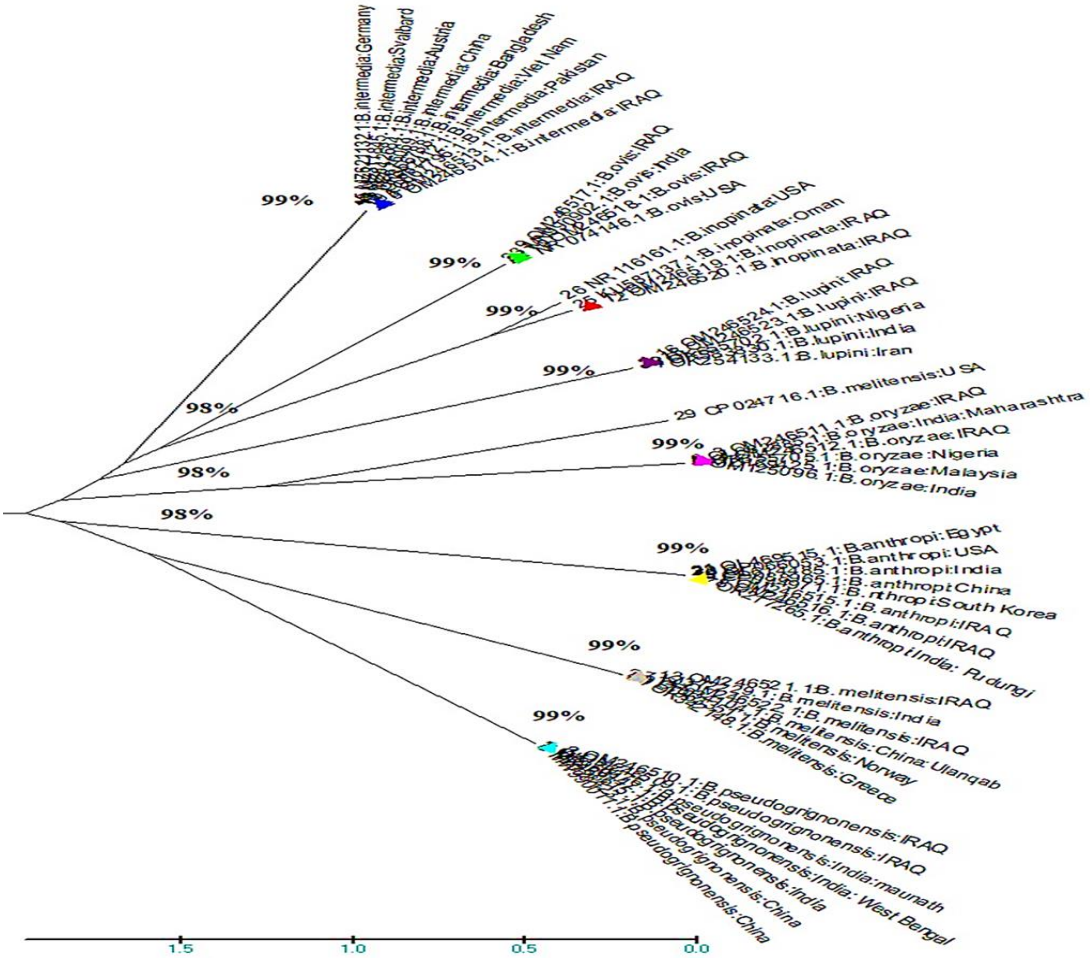

Figure 1-3. Evolutionary analysis (phylogenetic tree of *Brucella* isolates isolated from soil)) of *16S rRNA* gene sequences at compared of *B.intermedia* with different states. This figure(1-3) found that *B.intermedia* was nearest to Pakistan.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 29 December 2023 doi:10.20944/preprints202312.2292.v1

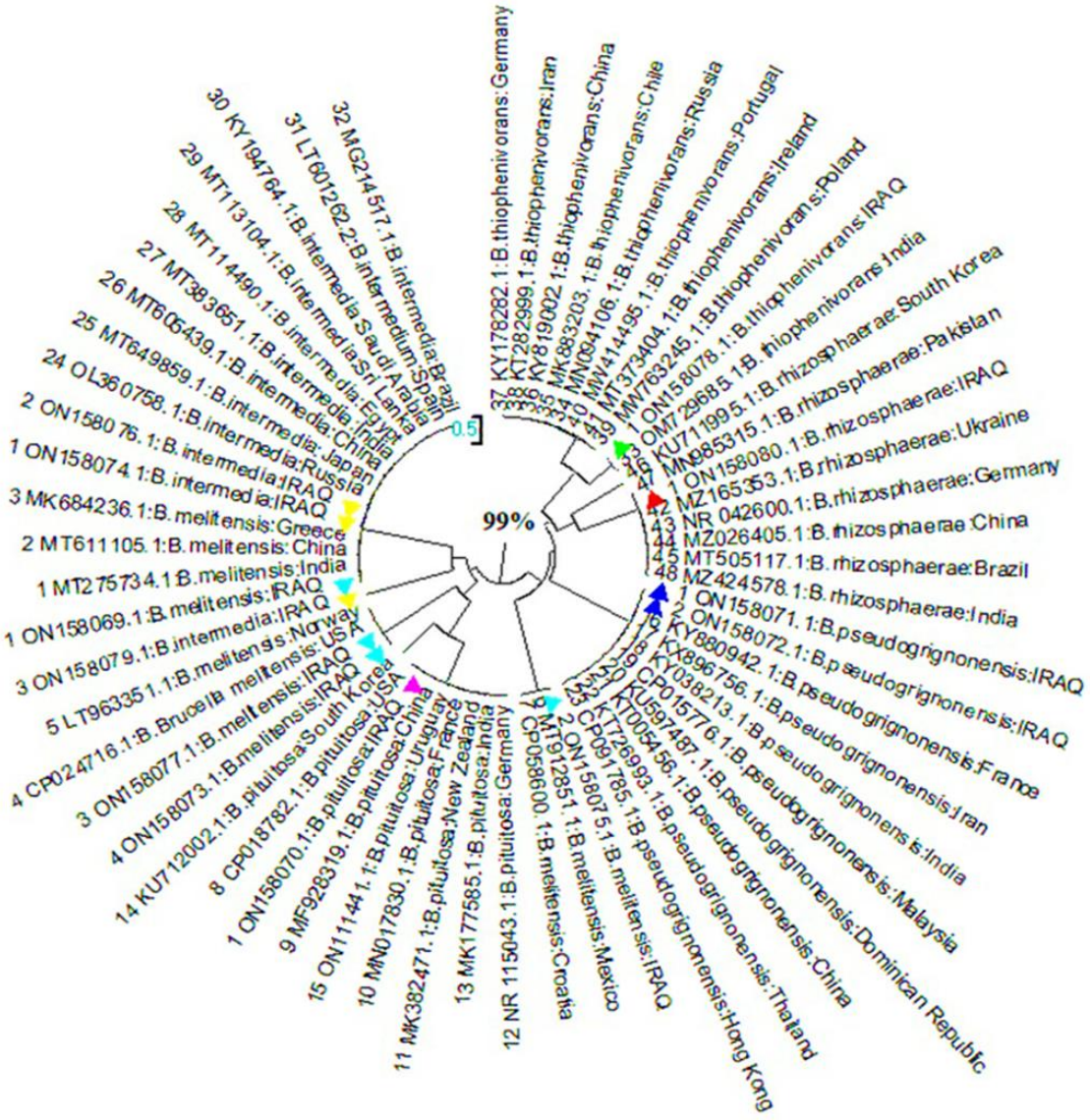

Figure (1-4). Evolutionary analysis (phylogenetic tree of *Brucella* isolates isolated from soil) of *16S rRNA* gene sequences of *B.intermedi* and *B.rhizosphaerae* at compared with different state. This figure(1-4) found that *B.intermedia* was nearest to Russia while *B.rhizosphaerae* was nearest to Ukraine and Pakistan.

The Relationship between the Environmental Factors and *Brucella* species

Our results proved existence relation between the presence of Brucella and the physical, chemical properties.

Table 1-6. The Relationship between the Four Seasons and the Physical, Chemical Parameters and the Percentage of *Brucella* species (Mean ± S.D). p≤0.05.

Seasons / *Brucella* species (%) and Environmental Parameters	Winter, 2021	Spring, 2021	Summer , 2021	Autumn, 2021

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 29 December 2023 doi:10.20944/preprints202312.2292.v1

***Brucella* per.(%)**	60±0.5[a]	78.13±0.87[b]	100±11.4 [c]	91.67±6.7 [d]
***B.intermedia* per. (%)**	0.00 [a]	50.00±3.68[b]	0.00 [a]	50.00±4.08[b]
***B.rhizosphaerae* per.(%)**	0.00 [a]	100.0±10.14[b]	0.00 [a]	0.00 [a]
Air Temp. (⁰C)	27.40 [a]	30.69±4.8 [b]	42.11±2.4 [c]	32.83±1.8[d]
Soil temp. (⁰C)	21.85 [a]	26.17±3.4[b]	37.11±3.1 [c]	23.01±7.3 [d]
pH	7.62±0.3 [a]	7.56±0.4 [a]	7.81±0.2 [b]	7.51±03. [a]
EC (µs/cm)	3957±14.5[a]	3768±22.6 [b]	4959±72.4 [c]	5123±18.1 [d]
TDS (mg/L)	2812±10.5[a]	2646±15.8 [b]	3549±21.6 [c]	3593±12.7 [d]
Salinity(‰)	2.53± 0.9[a]	2.47±0.3 [a]	3.17±0.7 [b]	3.28±1.1 [b]
TOC(%)	42.44±3.4 [a]	35.68± 2.3[b]	34.57±3.1 [c]	27.63±2.03[d]

This table(1-6) noted that proportion of *Brucella* presence in summer is higher that it is in relation with environmental parameters which include (Air and Soil temperature, pH, EC, TDS and Salinity) that are high , except TOC is low while proportion of *Brucella* 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 which is high**.** This indicated that variation of seasons affect to *Brucella* ratio in soil .

Relationship of the Heavy Metals with *Brucella* Species.

Our results confirmed existence relation between the presence of *Brucella* spp. and the heavy metals **,** as mentioned in table **(1-7).**

Table 1. The Relationship between the Four Seasons with *Brucella* species (%) and the Concentrations of Heavy Metals (Mean±S.D). p≤0.05.

Seasons / ***Brucella* species (%) and Heavy Metals**	**Winter, 2021**	**Spring, 2021**	**Summer, 2021**	**Autumn, 2021**
***Brucella* per.(%)**	60±0.5[a]	78.13±0.87[b]	100±11.4 [c]	91.67±6.7 [d]
***B.intermedia* per. (%)**	0.00 [a]	50.00±3.68[b]	0.00 [a]	50.00±4.08[b]
***B.rhizosphaerae* per.(%)**	0.00 [a]	100.0±10.14[b]	0.00 [a]	0.00 [a]
Cu (mg\kg)	12.5±0.41[a]	5.12±0.83 [b]	4.04±0.82 [b]	5.32±0.93 [b]
Fe(mg\kg)	412±0.83 [a]	304±0.82 [b]	315±0.46 [c]	396±2.89 [d]
Cd(mg\kg)	1.83±0.09[a]	0.52±0.02 [b]	1.18±0.01 [c]	1.72±0.09 [a]
Pb(mg\kg)	647±0.94 [a]	726±0.94 [b]	732±0.53 [c]	722±0.83 [d]

In the current reseach, it was observed that heavy metals affect the percentage of *Brucella* presence in soil, The high amounts of Cu ,Fe, Cd are in winter, where the percentage of *Brucella* is

 doi:10.20944/preprints202312.2292.v1

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* .

Discussion

A microbiological study is necessary for detecting cases of *Brucella* spp. because the symptoms are varied and non-specific, as shown by Bonaventura *et al*.,(2021[22] · Typical zoonotic illnesses *Brucella* spp. was the source of the disease brucellosis, which continues to pose a serious health risk in many underdeveloped nations worldwide[23]. The *16S rRNA* gene was the target of the PCR technique which was used to identify the bacteria in the samples[24]. Srinivasan *et al*., (2015)[25] who utilized 10 ng of genomic DNA extracted from each strain to amplify this gene. Sanger sequences have been created. Isolated 16S rRNA sequences with a genus and species name (Isolated named- strains 16S aligned. fasta) were obtained from the Greengenes database[26]. PCR was used to diagnose the risk of brucellosis in people who were exposed to animals at work. It is a more accurate for finding *Brucella* spp. To tackle this potentially hazardous zoonotic disease in Pakistan, especially where brucellosis is common in animals, there is an urgent need for more accurate and focused diagnostic tools like PCR[27], as mentioned in figures (1-1),(1-2). The *16S rRNA* gene was the target of the PCR technique which was used to identify the bacteria in the samples[28]. A final diagnosis of the condition is made using either serological or cultural methods, or both[29]. As a result, PCR-based approaches have improved in accuracy, sensitivity, speed, and ability to work with DNA rather than highly contagious live cultures as a result of their increased using for detecting and identifying *Brucella* species[30]. In particular, for slow-growing bacteria like *Brucella*, Rahman *et al*., (2014)[31] found that the PCR assays were efficient for speedy and precise diagnoses. It was shown by Manivannan *et al*., (2021)[32] to be a sensitive and specific approach for identifying *Brucella* spp.

In table (1-4) showed that all isolates of *B.intermedia* has significant differences ($p<0.05$) between genotype and environmental factors , This indicates presence of genetic variation in them. This implies the presence of genetic variation in them and suggests that these bacteria changed as a result of polymorphism caused by environmental indicators and heavy metals. This caused differences between them, allowing them to adapt to and live in harsh environments. In addition, to survive in this soil, its resistance to the poisonous heavy metals may be changed into new strains. The psychological differences amongst microorganisms are caused by genetic changes. while table (1-5) mentioned that *B.rhizosphaerae,* does not exhibit significant differences ($p>0.05$) between genotype and environmental parameters , This may be because *B. rhizosphaerae* is related to roots, *B. rhizosphaerae* is a gram-negative, oxidase-positive bacterium discovered in the rhizosphere of a potato in Austria[33].

There was many ways to become infected by the dangerous bacteria known as *Brucella*. Long-lasting resistance was possible in both inside and outside of mammalian hosts, even in harsh circumstances. It can linger in food for up to 15 months under adverse conditions such as acidity and temperatures ranging from 11 to 14 °C or for a few days if kept below 37 °C for up to two months in the winter and for only a few hours if exposed to direct sunshine, *Brucella* can also survive in contaminated manure and aborted infected feti[34]. *B. intermedia* is a bacterium in the genus *Brucella*[35]. Velasco *et al*., (1998)[36] described it initially. Only one case of cholangitis following liver transplantation has been reported in humans[37].

Exposure to Heavy metal persists and is rising in many regions of the world the fact that heavy metals were known to have several negative health impacts and that these effects could endure for a very long time. For ecological, evolutionary, nutritional and environmental reasons, heavy metals were significant environmental contaminants and their toxicity is a problem that is becoming more and more important[38,39].The oxidative degradation of biological macromolecules were mostly caused by heavy metal binding to DNA where it displaced original metals from their native binding sites and caused malfunction to cells and finally became toxic[40].

 doi:10.20944/preprints202312.2292.v1

The phylogenetic tree of *Ochrobactrum* spp. already shows the presence of the A44T-related strains in a separate branch[41]. Ramette *et al*., (2011)[42] based on the concatenated sequences of 16S rRNA and genes to acquire higher phylogenetic resolution within this group, which reveals that *O. rhizosphaerae* PR17T is the closest relative of A44T. The strain *Ochrobactrum* sp. was identified in the rhizosphere of a field-grown potato in Gelderland, the Netherlands. According to phylogenetic analysis based solely on the *16S rRNA* gene as in figures (1-3),(1-4).

Conclusions

1. In this research, it was noted that proportion of *Brucella* spp. presence in summer was high and also environmental parameters .
2. *Brucella* spp. survived in local soils due to environmental conditions that being variations in *Brucella* species, which led to a change in their properties and make it resistance. Recording new nucleotides sequencing in soil samples and presence of new isolates of *Brucella* in local soils samples. Registered new strains in soil were two new species of *Brucella* (*B. rhizosphaerae,* and *B. intermedia*).
3. Phylogenetic analysis of the 16S rRNA sequences noted that *B.intermedia* was nearest to Pakistan, and the other *B.intermedia* was nearest to Russia, while *B.rhizosphaerae* was nearest to Ukraine and Pakistan in animals soils samples, these stated consider one of the sources for the infection.

References

1. Leclercq, S. O.; Cloeckaert, A. and Zygmunt, M. S.(2020). Taxonomic organization of the family Brucellaceae based on a phylogenomic approach. *Frontiers in microbiology*. 10:3083.
2. Jones, J. D.; Treanor, J. J.; Wallen, R. L. and White, P. J. (2010). Timing of parturition events in Yellowstone bison Bison bison: implications for bison conservation and brucellosis transmission risk to cattle. *Wildlife Biology*.16(3):333-339.
3. Eilers, K. G.; Debenport, S.; Anderson, S. and Fierer, N. (2012). Digging deeper to find unique microbial communities: the strong effect of depth on the structure of bacterial and archaeal communities in soil. *Soil Biology and Biochemistry*. 50:58-65.
4. Seleem, M. N.; Boyle, S. M. and Sriranganathan, N.(2010). Brucellosis: a re-emerging zoonosis. *Veterinary microbiology*. 140(3-4):392-398.
5. Fouskis, I.; Sandalakis, V.; Christidou, A.; Tsatsaris, A.; Tzanakis, N.; Tselentis, Y. and Psaroulaki, A. (2018). The epidemiology of Brucellosis in Greece, 2007–2012: a 'One Health' approach. Transactions of The Royal Society of Tropical Medicine and Hygiene. 112(3):124-135.
6. Moreno, E. (2014). Retrospective and prospective perspectives on zoonotic brucellosis. *Frontiers in microbiology*. 5:213.
7. Abd El-Wahab, A. E. W.; Hegazy, Y.; Wael, F.; Mikeal, A.: Kapaby, A. F.; Abdelfatah, M. and Eltholth, M. M. (2019). Knowledge, attitudes and practices (KAPs) and risk factors of brucellosis at the human-animal interface in the Nile Delta, Egypt. *bioRxiv*. 607655.
8. Motsi, T. R.; Tichiwangana, S. C.; Matope, G. and Mukarati, N. L. (2013). A serological survey of brucellosis in wild ungulate species from five game parks in Zimbabwe: research communication. Onderstepoort Journal of Veterinary Research. 80(1):1-4.
9. Seiler, C. and Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in microbiology*. 3: 399.
10. Li, Y.; Li, X., Liang, S.; Fang, L. and Cao, W. (2013). Epidemiological features and risk factors associated with the spatial and temporal distribution of human brucellosis in China. BMC Infectious Diseases. 13(1):547.
11. Morales, J. R. A.(2013).Climate change, climate variability and brucellosis. *Recent patents on anti-infective drug discovery*.8(1):4-12.
12. Ahmed, R.; Muhammad, K.; Rabbani, M.; Khan, M. S.; Ali, M. A.; Naureen, S.; ... and Chang, Y. F. (2017). Phylogenetic Analysis of Soil Borne *Brucella* Species by Targeting Insertion Sequence 711 Element in Punjab, Pakistan. *International Journal of Agriculture and Biology*. 19(6):1457-1462.
13. Gaudette, H., Flight, W., Toner, L., Folger, D.(1974). An inexpensive titration method for the determination of organic carbon in recent sediments. *Journal of Sedimentary Petrology* .44: 249-253.
14. Sharidah M.M.A. (1999). Heavy metals in mangrove sediments of the United Arab Emirates shoreline (Arabian Gulf).Water Air Soil Pollut. 116:523-534.
15. American Public health Association (APHA). (1985). Standard Methods For Examination of Water and Wastewater, 20th Edition, American Public Health Association. Washington D. C.

16. Forbes, B. A.; Sahm, D. F. and Weissfeld, A. S. (2007). Bailey and Scotts' Diagnostic microbiology: A textbook for isolation and identification of pathogenic microorganisms. 12th. ed. *St Louis, Mosby*. 378.
17. Alton, G. G.; Jones, L. M.; Angus, R. D. and Verger, J. M. (1988). Bacteriological methods. In: Techniques for the brucellosis laboratory. Institute National de la Recherche Agronomique, INRA, Paris France. 34-60.
18. Dadar, M.; Alamian, S.; Behrozikhah, A. M., Yazdani, F.; Kalantari, A.; Etemadi, A. and Whatmore, A. M. (2019). Molecular identification of *Brucella* species and biovars associated with animal and human infection in Iran. Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. In *Veterinary research forum*. 10:4-315 .
19. Dadar, M. and Alamian, S. (2021). Identification of main *Brucella* species implicated in ovine and caprine abortion cases by molecular and classical methods. *Archives of Razi Institute*. 76(1):51.
20. Şahin, M. ; Unver ,A. and Otlu ,S. (2008). Isolation and biotyping of B. melitensis from aborted sheep foetuses in Turkey. *Bull Vet Inst Pulawy*. 52: 59-62.
21. Srinivasan, R.; Karaoz, U.; Volegova, M.; MacKichan, J.; Kato-Maeda, M.; Miller, S. and Lynch, S. V. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PloS one*. 10(2): e0117617.
22. Bonaventura, D.;Angeletti, G. S.; Ianni, A.; Petitti, T. and Gherardi, G. (2021). Microbiological laboratory diagnosis of human brucellosis: An overview. *Pathogens*.10(12):1623.
23. 23-Wang, Y.; Wang, Z.; Zhang, Y.; Bai, L.; Zhao, Y.; Liu, C.; ... and Yu, H. (2014). Polymerase chain reaction–based assays for the diagnosis of human brucellosis. *Annals of clinical microbiology and antimicrobials*. 13(1):1-8.
24. Hamdy, M. E. and Zaki, H. M. (2018). Detection of virulence-associated genes in Brucella melitensis biovar 3, the prevalent field strain in different animal species in Egypt. *Open Veterinary Journal*. 8(1):112-117.
25. Srinivasan, R.; Karaoz, U.; Volegova, M.; MacKichan, J.; Kato-Maeda, M.; Miller, S. and Lynch, S. V. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PloS one*. 10(2): e0117617.
26. DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., ... & Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology*, *72*(7), 5069-5072.
27. Asif, M.; Waheed, U.; Farooq, M.; Ali, T. and Khan, Q. M. (2014). Frequency of Brucellosis in High Risk Human Groups in Pakistan Detected through Polymerase Chain Reaction and its Comparison with Conventional Slide Agglutination Test. *International Journal of Agriculture & Biology*. 16(5).
28. 28-Hamdy, M. E. and Zaki, H. M. (2018). Detection of virulence-associated genes in Brucella melitensis biovar 3, the prevalent field strain in different animal species in Egypt. *Open Veterinary Journal*. 8(1):112-117.
29. Etman, R. H.; Barsoum, S. A.; Ibrahim, I. G. A. ; El-Ashmawy, W. R. and Abou-Gazia, K. A. (2014). Evaluation of efficacy of some serological tests used for diagnosis of brucellosis in cattle in Egypt using latent class analysis. *Sokoto Journal of Veterinary Sciences*. 12(2):1-7.
30. Moussa, I. M.; Omnia, M. E.; Amin, A. S. and Selim, S. A. (2011). Evaluation of the currently used polymerase chain reaction assays for molecular detection of Brucella species. *African Journal of Microbiology Research*. 5(12):1511-1520.
31. Rahman, M. S.; Sarker, M. A. S.; Rahman, A. K. M. A. ; Sarker, R. R.; Melzer ,F. ; Sprague, L. D. and Neubauer, H. (2014) . The prevalence of *Brucella abortus* DNA in seropositive bovine sera in Bangladesh. *Afr. J. Microbiol. Res*. 8: 3856-3860..
32. Manivannan, K.; Mahmoud, S. M.; Ramasamy, M.; Shehata, A. A.; Ahmed, H.; Solaimuthu, C. and Dhandapani, K. (2021). Molecular detection of brucellosis in dromedary camels of Qatar by real-time PCR technique. *Comparative Immunology, Microbiology and Infectious Diseases*.78:101690.
33. Kämpfer, P.; Sessitsch, A.; Schloter, M.; Huber, B.; Busse, H. J. and Scholz, H. C. (2008). *Ochrobactrum rhizosphaerae* sp. nov. and Ochrobactrum thiophenivorans sp. nov., isolated from the environment. *International journal of systematic and evolutionary microbiology*. 58(6):1426-1431.
34. 34-Lucero, N.; Tenenbaum, M.; Jacob, N.; Escobar, G.; Groussaud ,P. and Whatmore, A.(2010).Application of variable number of tandem repeats typing to describe familial outbreaks of brucellosis in Argentina. J Med Microb. 59:648-52.
35. Liu, D. (Ed.). (2011). Molecular detection of human bacterial pathogens. CRC press.
36. Velasco, J.; Romero, C.; Lo´ pez-Gon˜ i, I.; Leiva, J.; Dı´az, R. and Moriyo´ n, I. (1998). Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol*. 48:759-768.
37. Mo¨ller, L. V.; Arends, J. P.; Harmsen, H. J.; Talens, A., Terpstra, P. and Slooff, M. J. (1999). Ochrobactrum intermedium infection after liver transplantation. *Journal of clinical microbiology*. 37(1):241-244.
38. Jaishankar, M.; Tseten, T.; Anbalagan, N.; Mathew, B. B. and Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary toxicology*. 7(2):60.

39. Nagajyoti, P. C.; Lee, K. D. and Sreekanth, T. V. M. (2010). Heavy metals, occurrence and toxicity for plants: a review. *Environmental chemistry letters*. 8(3):199-216.
40. Flora, S. J. S.; Mittal, M. and Mehta, A. (2008). Heavy metal induced oxidative stress & its possible reversal by chelation therapy. *Indian Journal of Medical Research*. 128(4):501.
41. Kämpfer, P.; Scholz, H. C.; Huber, B.; Falsen, E. and Busse, H. J. (2007). Ochrobactrum haematophilum sp. nov. and Ochrobactrum pseudogrignonense sp. nov., isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*. 57(11):2513-2518.
42. Ramette, A; Frapolli, M; Le Saux ,F.M; Gruffaz ,C.; Meyer, J.M, De´fago G;… *et al*.(2011). *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. Syst Appl Microbiol. 34: 180-188.