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RISK FACTORS ASSOCIATED WITH THE PREVALENCE OF SOIL BORNE *BRUCELLA* SPECIES DETECTED BY METAGENOMICS APPROACH IN PUNJAB, PAKISTAN

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ABSTRACT

Brucella species infect animals and also have zoonotic effect. In the present study, risk factors associated with existence of *Brucella* species in soil were investigated by using metagenomics approach. By using grid based sampling strategy, soil samples (n= 1280) were collected from 256 villages (5 samples/village) of nine districts of Punjab including Lahore, Faisalabad, Sheikhupura, Sargodha, Dera Ghazi Khan, Chakwal, Sahiwal, Gujranwala and Attock. Physical risk factors such as distance near to water irrigation source, distance near to animal market and animal density per village were significantly associated (OR>1) with the prevalence of soil borne *Brucella* species while other risk factors such as distance from main road and No. of houses per village were less associated (OR<1). Association of chemical risk factors was analyzed by mann-whitney test assuming level of significance of 0.1 and 90% confidence interval. Manganese, lead, zinc, silt, clay, organic matter and soluble salts are significantly associated (P<0.1) with the prevalence of *Brucella* species while other risk factors including pH, sand, moisture, nitrogen, phosphorus, nickel, cadmium, copper, chromium, iron, calcium, magnesium, sodium and potassium were not associated (P>0.1). In conclusion, physical and chemical risk factors showed association with prevalence of soil borne *Brucella* species through metagenomics analysis.

Key words: *Brucella*, DNA, risk factors, PCR, Soil, Metagenomics.

INTRODUCTION

The genus *Brucella* is classified as category B pathogen by Centre of Disease Control and Prevention (CDC) (Manual, 2005) and is placed in Risk group III by World Health Organization (WHO). There is occupational exposure of *Brucella* species to shepherds, veterinarians, farmers, abattoir workers and laboratory personnel who handle infected animals and contaminated animal products (Kosgei, 2016). In addition, *B. abortus* and *B. melitensis* are important food-borne pathogens that may be acquired by consuming raw milk and milk products such as soft cheese (Leclerc *et al.*, 2002, Kuplulu & Sarimehmetoglu, 2004). *B. melitensis* is the most virulent species for humans and accounts for the majority of cases of human brucellosis (Leclerc *et al*., 2002).The affected animals show the sign and symptoms of abortions, retained placenta, reduced milk yield, production of weak calves, orchitis, epididymitis and infertility (Nicoletti, 1980, Silva *et al.*, 2000, Taleski *et al.*, 2002, Thomson & Bastos, 1994, Kumi-Diaka *et al.*, 1980).The contaminated soil by *Brucella* species (which are excreted through vaginal discharge, aborted fetuses, milk and semen of infected animals) is the source of infection to humans. Humans are mostly infected with *B. melitensis*, then *B. abortus* and at last *B. suis* in order of their infectivity (Acha & Szyfres, 2003). Transmission occurs through direct contact, inhalation, penetration through broken skin, and consumption of unpasteurized milk (Olsen & Palmer, 2014). Person to person transmission is very rare (Meltzer *et al.*, 2010) and chances of accidental infection with vaccines are also very low (Ashford *et al.*, 2004, Strausbaugh & Berkelman, 2003). *Brucella* species are sensitive to heat and can survive for several weeks in water (Franz *et al.*, 1997). The survival ability of *B. abortus* in the environment is a great risk factor for transmission of brucellosis to the relevant hosts (Kuzdas & Morse, 1954). *Brucella* species survive in soil and dust for many weeks (Franz *et al*., 1997). *B. abortus* survive in aborted fetus up to 135 days in winter, more than 2 months in cool environment (Manual, 1998) and 6 months in shaded fetus (Wray, 1975). Sunlight and temperature has negative effect on the survival of *B. abortus* in the environment (Jones *et al.*, 2010). *B. abortus* can survive up to 66 days in wet soil, when humidity increased up to 90%, it can survive for 48 to 73 days and less than 4 days in dry soil (Nicoletti, 2001). Prevalence of *Brucella* species in the soil is one of the risk factors for its spread to animals and humans. There are many factors associated with prevalence of *Brucella* species (Seleem *et al.*, 2010). The present study investigates the association of soil risk factors (physical and chemical) with the prevalence of *Brucella* species through metagenomics approach.

MATERIALS AND METHODS

Study Area and Sample Collection: The soil samples were collected from nine districts of Punjab. To select the district for sampling, a map of Punjab was scanned on 600 dpi resolution and geo-referenced with geographic coordinate system. All the geographical coordinates of the samples were recorded using GPS receiver (Garmin, Dakota U.S.A.) and mapped using recorded data and sequence. ArcGIS 10.1 (Esri, California) software was used to develop maps to see the spatial distribution of *Brucella* species in nine districts. Soil samples (n=1280) were collected from 10 % villages (n= 256 villages) of nine districts of Punjab. Personal protective equipments (PPEs) were used for soil sampling. From each village five samples were collected. Samples were collected in such a way that four samples (n=4) were taken from each corner of the village where animals and humans were in close proximity while the fifth sample was taken as a control from outside the village, the soil where human and animal populations were not frequently interacting. The soil samples were taken three inches below the ground surface.

DNA Extraction from Soil Samples: Each of the soil samples was measured (0.25g) out through a weighing balance (Local Brand) and was labeled. The samples were processed for metagenomic DNA extraction using **Power Max™** Soil DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA) as per manufacturer's recommendations. After extraction, the quality of DNA was determined using Nano Drop 1000 spectrophotometer (Thermo scientific, USA) following the methods of Desjardins and Conklin (2010).

Conventional Multiplex PCR: All DNA samples were analyzed for *Brucella* species (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis*) through Conventional Multiplex PCR (CFX96 ™ Real-Time PCR Detection Machine (BIO-RAD, U.S.A.) as described by Ali *et al.* (2014). The Species specific primers were designed against sequences present in the GenBank, National Centre for Biotechnology Information (NCBI) using Primer 3 software and Insilico PCR web facility (Rozen & Skaletsky, 1999) using the reference sequences of these genes. The composition of reaction mixture and PCR conditions were optimized and followed as described by Ali *et al*. (2014). The PCR products were checked on 2% agarose gel by doing electrophoresis for 90 min at 105 volts along with DNA ladder (100bp). The gel was visualized under UV light using a gel documentation system (Syngene, UK).

Physical Risk Factors: The soil samples were collected from the villages and a questionnaire was also filled containing information about physical risk factors such as distance from animal market, distance from main road, distance from water irrigation source, animal density of the area and number of houses per village. Regarding the distance from animal market, the samples were collected either >1km from market or <1km distance near to animal market, regarding the distance from main road, the soil samples were collected either >500 meters away from the road or <500 meters near to main road, regarding water irrigation source, samples were collected either <100 meters near to water source or >100 meters away from water bodies, regarding animal density, samples were collected from the area having >1000 animals or the area having <1000 animals and regarding the number of houses, the soil samples were collected from the area where number of houses was >300 or <300 per village.

Chemical Risk Factors: A total of 27 soil samples (each of positive and negative sample of *Brucella* species from the same district) were analyzed for physical and chemical properties including pH, moisture, total soluble salts, phosphorous, copper, chromium, nickel, manganese, cobalt, lead, cadmium, iron, sodium, potassium, calcium, magnesium, nitrogen and organic matter. The physical properties of soil samples from the study area were carried out in Phytohormones Lab, Department of Plant Sciences, Quaid-e-Azam University, Islamabad and soil &water testing Laboratory Rawalpindi. Chemical risk factors such as soil pH was measured as described by McKeague (1978), moisture contents by Topp *et al.* (1980), soil texture was determined as described byTaubner *et al.* (2009)and total soluble salts were measured as described by Rhoades (1982).The macronutrients such as phosphorus (P) was determined by following protocol described by Fixen *et al.* (1990), nitrogen (N), sodium (Na), potassium (k), calcium (Ca) and magnesium (Mg) was measured as described by Kim *et al.* (2007).The micronutrients such as copper (Cu), chromium (Cr), nickel (Ni), manganese (Mn), cobalt (Co), lead (Pb), cadmium (Cd) and iron (Fe) were determined as described by Soltanpour and Schwab (1977).

Data Analysis: Results of all risk factors were compiled in a single Microsoft Excel spread sheet. Results of physical risk factors were analyzed by calculating odd ratio values using openepi software (version 2.3.1). Odd ratio result was interpreted as if OR=1 then there is no association between exposure and its outcome, if OR>1 then there is high association between exposure and its outcome and if OR<1 then there is very less effect of exposure with its outcome. The chemical risk factors were analyzed by Mann-Whitney test through statistical software SPSS (version 20.0; SPSS Inc., Chicago, IL) using 90% confidence interval and 10% level of significance. Normality of data was also checked by using Shapiro-wilk test.

RESULTS

DNA of two species *B. abortus* and *B. melitensis* was detected through conventional multiplex PCR of total genomic DNA of soil. Out of nine districts, only three districts Sheikhupura, Faisalabad and Sargodha were positive for soil borne *Brucella* species. All districts showed a significant result for detecting *Brucella* species (χ^2 =54.505, df=8, p<0.05). In District Sheikhupura, five soil samples were positive for the presence of *B. abortus* and two samples were positive for *B. melitensis*. The soil samples from villages Abdalia, Thatta Alyas, Muradpur, MirzaVirkan and Kilay were positive for *B. abortus* and two villages Chambal and Chak Phannar were positive for soil borne *B. melitensis*. The positive samples were collected <1km near to animal market, >500 meters away from main road, <100 meters near to water irrigation system, >1000 animals were housed there and population of the villages was >300 houses per village. Odd ratios for the risk factors were determined through Openepi software (version 3.2.1). The risk factors such as distance<1km near to animal market, distance <100 meters near to water irrigation source and animal density >1000 animals per village were significantly more associated (OR>1) with the existence of soil borne *Brucella* species while other risk factors such as distance from main road and no. of houses per village were less associated (OR<1) with the existence of DNA of *Brucella* species (Table 1&2). In District Faisalabad, nine soil samples from villages Chak 106 (2 samples), Chak 142 GB, Chak 228 GB, Chak 507 GB (2 samples), Chak 86 JB and Chak 88 JB (2 samples) were positive for *B. abortus* and these positive samples were collected <1km near to animal market, <500 meters near to main road, <100 meters near to water irrigation source,>1000 animals were housed per village and the population of the villages was >300 houses per village. The risk factors such as the distance (<100 meters) from water irrigation source, distance (<1km) from animal market and animal density (>1000 animals) per village were significantly more associated (OR>1) with the existence of soil borne *B. abortus* while the risk factors such as distance from main road and no. of houses (>300 or <300) per village were significantly less associated (OR<1) with the existence of *B. abortus* in the soil (Table 1). In District Sargodha, nine soil samples were positive for the presence of *B. abortus* and two were positive for soil borne *B. melitensis*. The soil samples from villages Chak 16/sb, Chak 15/sb, Moazmabad, Chak 3/sb (2 samples), Rawana, Chani dal, Chak Khana and Laluwalai were positive for *B. abortus* and the villages Dera madansani and Abdal were positive for soil borne *B. melitensis*. The soil samples were collected <1km near to animal market, >500 meters away from main road,<100 meters near to a water irrigation source, >1000 animals were housed per village and human population per village was >300 houses. The risk factors such as distance (<100 meters) from water irrigation source, distance (<1Km) from animal market and animal density (>1000) per village were significantly more associated (OR>1) with the existence of soil borne *Brucella* species while the risk factors such as distance from main road and no. of houses per village were significantly less associated (OR<1) with the existence of *Brucella* species in the soil (Table 1&2). Since soil samples collected from villages of districts Gujranwala, Sahiwal, Chakwal, D.G Khan, Attock and Lahore, did not show any positive results, therefore, odds ratio for the associations of risk factors could not be calculated. The chemical risk factors were analyzed by mann-whitney test with 90% confidence interval and 0.1 level of significance in SPSS (version 20.0). The Chemical risk factors such as manganese (Mn), lead (Pb), zinc (Zn), silt, clay and organic matter are positively associated (P<0.1) with the existence of *B. abortus* (Table 3) while one risk factor that is soluble salts showed a positive association (P<0.1) with the existence of soil borne *B. melitensis* (Table 4). Other risk factors including pH, sand, moisture, nitrogen, phosphorus, nickel, cadmium, copper, chromium, iron, calcium, magnesium, sodium and potassium were not associated (P>0.1) with existence of soil borne *Brucella* species.

Table 1. Association of risk factors with presence of soil borne *B. abortus* in samples from Sheikhupura, Faisalabad and Sargodha districts

Criteria	Sheikhupura			Faisalabad			Sargodha		
	+ve	-ve	OR (95% CI)	+ve	-ve	OR (95% CI)	+ve	-ve	OR (95% CI)
Distance from Animal Market									
<1 kilometre	3	42	3.5 (0.5641, 21.71)	7	67	4.127 (0.8293,	6	71	1.831
>1kilometre	2	98		2	79	20.54)	3	65	(0.4399, 7.621)
Distance from Main Road									
> 500 meters	1	52	0.4231	1	49	0.2474	3	69	0.4855
< 500 meters	4	88	(0.04605, 3.887)	8	97	(0.03009, 2.035)	6	67	(0.1167,2.021)
Distance from Water Irrigation Source (Canal/stream/drain)									
< 100 meters	2	49	1.238	8	66	9.697	5	62	1.492

> 100 meters	3	91	(0.2001, 7.66)	1	80	(1.183, 79.51)	4	74	(0.384, 5.797)
Animal Density									
>1000 animals	2	51	1.163	6	53	3.509	6	47	3.787
< 1000 animals	3	89	(0.1881, 7.194)	3	93	(0.843, 14.61)	3	89	(0.9062, 15.83)
No of houses/village									
> 300	3	98	0.6429	3	72	0.5139	3	88	0.2727
< 300	2	42	(0.1036, 3.988)	6	74	(0.1238, 2.133)	6	48	(0.0652, 1.139)

Table 2. Association of risk factors with presence of soil borne *B. melitensis* in samples from Sheikhupura and Sargodha districts

Criteria	Sheikhupura			Sargodha		
	+ve	-ve	OR (95% CI)	+ve	-ve	OR (95% CI)
Distance from Animal Market						
<1 kilometre	1	56	1.554 (0.0952,	1	30	3.767
> 1kilometre	1	87	25.34)	1	113	(0.2289,61.99)
Distance from Main Road						
> 500 meters	0	53	Not Possible	0	47	Not Possible
< 500 meters	2	90		2	96	
Distance from Water Irrigation Source (Canal/stream/drain)						
< 100 meters	1	65	1.2	1	66	1.167
> 100 meters	1	78	(0.07362, 19.56)	1	77	(0.07158,19.01)
Animal Density						
> 1000 animals	1	54	1.648	1	64	1.234
< 1000 animals	1	89	0.101, 26.89)	1	79	(0.07573,20.12)
No of houses/village						
> 300	1	78	0.8333	1	92	0.5543
< 300	1	65	(0.0511, 13.58)	1	51	(0.0339, 9.05)

Table 3. Chemical risk factors associated with prevalence of *B. abortus* in soil.

	Test Statistics[a]			
	Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)
pH	1305.000	8931.000	-.588	.556
Sand	1178.500	8804.500	-1.270	.204
Silt	976.000	8602.000	-2.357	.018
Clay	714.000	990.000	-3.765	.000
Soluble salts	1353.000	8979.000	-.330	.741
Moisture	1312.000	8938.000	-.551	.582
Organic matter	951.000	8573.000	-2.490	.013
N	1133.500	8759.500	-1.510	.131
P	1303.000	1579.000	-.599	.549
Ni mg/kg	1223.500	1499.500	-1.026	.305
Cd mg/kg	1265.000	1541.000	-.803	.422
Cu mg/kg	1384.500	8887.500	-.100	.920
Cr mg/kg	1141.500	1417.500	-1.467	.142
Mn mg/kg	904.000	8530.000	-2.742	.006
Fe mg/kg	1119.500	8745.500	-1.585	.113
Ca mg/kg	1406.000	9032.000	-.046	.964
Mg mg/kg	1324.500	8950.500	-.483	.629
Pb mg/kg	1095.500	1371.500	-1.714	.087
Na mg/kg	1228.000	8854.000	-1.002	.316
Zn mg/kg	1094.500	1370.500	-1.719	.086
K mg/kg	1378.000	9004.000	-.196	.845

a. Grouping Variable: *B. abortus*

Table 4. Chemical risk factors associated with prevalence of *B. melitensis* in soil.

Test Statistics[a]				
	Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)
pH	188.000	10341.000	-1.151	.250
Sand	268.000	10421.000	-.192	.848
Silt	162.500	10315.500	-1.457	.145
Clay	156.000	166.000	-1.535	.125
Soluble salts	98.000	108.000	-2.231	.026
Moisture	256.500	266.500	-.330	.742
Organic matter	236.000	10389.000	-.575	.565
N	231.000	10384.000	-.635	.525
P	254.500	264.500	-.354	.724
Ni mg/kg	276.000	10429.000	-.096	.924
Cd mg/kg	249.500	10402.500	-.414	.679
Cu mg/kg	191.500	201.500	-1.093	.275
Cr mg/kg	275.500	10428.500	-.102	.919
Mn mg/kg	234.500	10387.500	-.593	.553
Fe mg/kg	244.000	10397.000	-.480	.632
Ca mg/kg	246.500	10399.500	-.450	.653
Mg mg/kg	183.500	193.500	-1.205	.228
Pb mg/kg	229.500	10382.500	-.653	.513
Na mg/kg	150.500	10303.500	-1.600	.109
Zn mg/kg	239.500	249.500	-.533	.594
K mg/kg	206.500	216.500	-.929	.353

a. Grouping Variable: *B. melitensis*

DISCUSSION

Brucella species infect animals and also have zoonotic effect. It is a laboratory acquired infection (Collins, 1988), so persons working in laboratory are at high risk of exposure with *Brucella* species. In laboratory, diagnosis is usually done through serological tests which give cross reactivity with other species so gold standard for diagnosis is culture method that require biosafety level 3 (BSL3) laboratory for its handling. In the present study, PCR of metagenomic DNA (culture-independent approach) was used as it is highly sensitive (97%) and specific, gives high-throughout simultaneous detection of several samples, has rapid turnaround time and poses less threat to laboratory workers as compared to culture-dependent methods.

Soil of three districts Faisalabad, Sheikhupura and Sargodha was positive for DNA of *B. abortus* and *B. melitensis*. Bacterial DNA is released in to the environment actively from living cells or passively from dead cells. There is autolysis of bacterial cells and release of intracellular contents including DNA (Palmen & Hellingwerf, 1995, Palmen & Hellingwerf, 1997). Bacterial DNA persist in soil for a long period of time depending upon the availability of degradation enzymes and type of soil (DeSalle *et al.*, 1992, Smith *et al.*, 2001).

In the soil DNA molecules adsorb to soil colloids and minerals that prevent its enzymatic degradation particularly from DNases (Romanowski *et al.*, 1991). Divalent cations promotes DNA adsorption and form complexes with phosphate backbone of DNA and enhance its adsorption (Paget *et al.*, 1992). In the present study, chemical risk factors such as manganese (Mn), lead (Pb) and zinc (Zn), which are divalent cations and are positively associated with the existence of soil borne *Brucella* species. These mineral elements enhanced DNA adsorption with soil particles, prevented DNase degradation, DNA persisted there and detected through PCR.

Sodium (Na) and potassium (k) are not significantly associated with the prevalence of *Brucella* species in the present study and according to Romanowski *et al*. (1991) the adsorption of DNA is affected by the concentration and valency of cations, divalent cations (Zn^{+2}&Mn^{+2}) are 100 folds more effective than monovalent cations (Na^{+}, K^{+}).

In the present study, clay and organic matter of the soil were found to be positively associated with the prevalence of *Brucella* species but the soil pH, sand and silt did not show significant association. DNA adsorption on the clay was observed to be highest (Lorenz & Wackernagel, 1994) than sand (100 folds lower than clay) and silt (intermediate between sand and clay) (Levy-Booth *et al.*, 2007).Therefore the ratio of silt, sand and clay in the soil will affect DNA adsorption (Blum *et al.*, 1997). Acidic soil supports more adsorption of DNA than alkaline soil (Stotzky, 2000). In the present study cadmium (Cd) and chromium (Cr) elements in soil did not show significant association with the prevalence of *Brucella* species. The concentration of cadmium in earth

crust is 0.1 mg/kg while the concentration of chromium in the environment has increased due to its release from chemical industries and refectories (Zayed *et al.,* 2003). It was observed in the *in vitro* study that 0.1 to 10mM concentration of cadmium is sufficient to induce cytotoxicity in the cells and is also responsible for DNA damage (Tsuzuki *et al.*, 1994, Mukherjee & Das, 2002). Chromium in hexavalent form [Cr (VI)] is cytotoxic (Tchounwou *et al.*, 2012) and breaks DNA strands and causes DNA fragmentation (Patlolla *et al.*, 2009). According to Trevors (1996) presence of magnesium (Mg) and calcium (Ca) in the soil, lowers the adsorption efficiency of DNA, sodium and magnesium, both are in competition to lower DNA adsorption and attachment efficiencies (Nguyen & Chen, 2007). In the present study, soil elements such as nitrogen (N), phosphorus (P), iron (Fe), copper (Cu) and nickel (Ni) did not show positive association with the prevalence of *Brucella* species. Heavy metals such as cadmium, arsenic, chromium, copper, lead, mercury, nickel, selenium, molybdenum, zinc, titanium and antimony are added in to the soil by animal's manure, bird's droppings, dung and decayed organic matter (Basta *et al.*, 2005). In some of the villages, farmers are used to apply animal's manure and droppings of poultry birds in their fields as fertilizer for the crops but in poultry feed copper and zinc are added therefore it causes metal contamination of the soil (Chaney & Oliver, 1996). The soil contaminated with metals such as nickel, cadmium and chromium degrade DNA and increase DNases activity (Schmidt, 1996). The soil of the villages of district Faisalabad positive for *Brucella* species is located with latitude 31.3235° N and longitude 73.1822° E and at elevation 186 meters above sea level. The positive soil of these villages is clay and sandy type and this soil supports DNA stability up to months as described by Romanowski *et al.* (1993). In district Sheikhupura, the villages positive for *Brucella* species are situated with latitude 31.7166 ′N and longitude 73.9850 ^{0}E. The soil of positive villages is loamy sand type and according to Recorbet *et al.* (1993) this type of soil supports DNA stability up to months. The soil of the villages of district Sargodha is positive for *Brucella* species because two rivers flow near city area and the soil of this district is of sandy loam type and chances of DNA survival and stability are very high in this type of soil as described by Selenska and Klingmüller (1992). Physical risk factor that is distance (<1 Km) near to animal market showed positive association with the prevalence of *Brucella* species, as in animal market, animals are brought from different areas by animal sellers. *Brucella* infected animals in the animal market are not only the source of infection to healthy ones but also shed *Brucella* organisms in the environment that contaminate soil (Musallam *et al.*, 2015) and in the soil *Brucella* species survive for a long time and in the present study DNA is detected from these soil samples through PCR. In the villages, usually brucellosis suspected animals are used to sell to a buyer or in animal market. There is widespread trade of *Brucella* infected animals and it is the major contributor factor for high endemicity of the brucellosis among the ruminants in above mentioned districts where there are no restrictions on animal's movements between districts and governorates. Distance (<100 meters) from irrigated water also shows positive association with DNA existence in soil. *Brucella* species survive in wet soil up to 66 days (humidity up to 90%) and their survival is <4 days in dry soil (Nicoletti, 2001). Animal density >1000 animals per village also showed positive association with existence of *Brucella* species. Animal density is positively associated with the frequency of Brucellosis (Haghdoost *et al.*, 2007). In the conclusion, Physical risk factors such as distance near to animal market, animal density and distance near to water irrigation source, and chemical risk factors such as manganese, lead, zinc, silt, soluble salts, organic matter and clay are associated with the prevalence of *Brucella* species in the soil and is a threat for animal and human health.

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