



# Cross Sectional Study and Risk Factors Analysis of *Francisella tularensis* in Soil Samples in Punjab Province of Pakistan

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Tularemia is an endemic zoonotic disease in many parts of the world including Asia. A cross-sectional study was conducted to determine genome-based prevalence of *Francisella tularensis* (*Ft*) in soil, assess an association between its occurrence in soil and likely predictors i.e., macro and micro-nutrients and several categorical variables, and determine seroconversion in small and large ruminants. The study included a total of 2,280 soil samples representing 456 villages in eight districts of the Punjab Province of Pakistan followed by an analysis of serum antibodies in 707 ruminants. The genome of *Ft* was detected in 3.25% ( $n = 74$ , 95% CI: 2.60–4.06) of soil samples. Soluble salts (OR: 1.276, 95% CI: 1.043–1.562,  $p = 0.015$ ), Ni (OR: 2.910, 95% CI: 0.795–10.644,  $p = 0.106$ ), Mn (OR: 0.733, 95% CI: 0.565–0.951,  $p = 0.019$ ), Zn (OR: 4.922, 95% CI: 0.929–26.064,  $p = 0.061$ ) and nutrients clustered together as PC-1 (OR: 4.76, 95% CI: 2.37–9.54,  $p = 0.000$ ) and PC-3 (OR: 0.357, 95% CI: 0.640,  $p = 0.001$ ) were found to have a positive association for the presence of *Ft* in soil. The odds of occurrence of *Ft* DNA in soil were higher at locations close to a water source, including canals, streams or drains, [ $\chi^2 = 6.7$ , OR = 1.19, 95% CI: 1.05–3.09,  $p = 0.004$ ] as well as places where animals were present [ $\chi^2 = 4.09$ , OR = 2.06, 95% CI: 1.05–4.05,  $p = 0.02$ ]. The seroconversion was detected in 6.22% ( $n = 44$ , 95% CI: 4.67–8.25) of domestic animals. An occurrence of *Ft* over a wide geographical region indicates its expansion to enzootic range, and demonstrates the need for further investigation among potential disease reservoirs and at-risk populations, such as farmers and veterinarians.

**Keywords:** *Francisella tularensis*, soil, domestic animals, Punjab province, Pakistan

## INTRODUCTION

Tularemia is caused by the bacterium *Francisella tularensis* (*Ft*), a category A classified select agent by the Center for Disease Control and Prevention (<https://www.selectagents.gov/SelectAgentsandToxinsList.html>). *Francisella tularensis* (*Ft*) is a pleomorphic Gram-negative intracellular bacterium (Schulert and Allen, 2006) that has zoonotic implications across many parts of the globe (Oyston, 2008; Vogler et al., 2009). Four subspecies, namely, *tularensis*, *mediasiatica*, *holarctica*, and *novicida*, have been identified (Sjöstedt, 2005; Champion et al., 2009; Penn, 2015). The presence of *Ft* subspecies *holarctica* has been reported in Europe and Asia, whereas *Ft* subspecies *tularensis* has been reported in North America (Garaizar et al., 2006). Among the Asian countries, most of the outbreaks and cases have been reported in Turkey (607 cases in 2012), China (31 cases in 1986), and Iran (36 cases in 2013) (Esmaili et al., 2014; Gürçan, 2014). The organism has a broad and complex host distribution that includes vertebrates, invertebrates, and environmental matrices such as soil, aerosols, and water (Kuske et al., 2006; Silvestri et al., 2016). Humans can acquire infection through inhalation, an arthropod bite, ingestion of contaminated food, or water, as well as through contact with infected tissues or fluid from animals (Oyston, 2008; Ulu-Kilic and Doganay, 2014; Silvestri et al., 2016). Individuals living in rural areas or those in close proximity to animals/disease reservoirs, especially farmers and veterinarians, are considered the most at-risk population for tularemia (Lévesque et al., 1995; Ulu-Kilic and Doganay, 2014). Though glandular, oculo-glandular, ulcero-glandular, typhoidal, and pneumonic symptoms are common in affected humans, the clinical signs and severity of disease depend on the entry route and infectivity dose (<10 CFU) (Helvacı et al., 2000; Bossi et al., 2004; Pechous et al., 2009). Just as in humans, clinical signs in animals are varied. Cats are more susceptible than dogs and remain mostly in non-clinical form however in some cases, symptoms may include fever, lymphadenopathy, anorexia, oral ulceration, hepatosplenomegaly, and dehydration (Gliatto et al., 1994; Woods et al., 1998).

Different subspecies of *Ft* have been reported worldwide with varying geographic distribution and disease potential. Occurrence of tularemia caused by the most virulent type (*F. tularensis*: biovar A) has been reported only from North America, while cases caused by the less virulent type (*F. holarctica*: biovar B) have been observed in Europe, North America, and Asia (Garaizar et al., 2006; Oyston, 2008; Esmaili et al., 2014; Ulu-Kilic and Doganay, 2014). However, there is a paucity of data on the enzootic range of *Ft* in Pakistan. Since *Ft* has the potential to survive and persist in the environment for a longer period of time (Sjöstedt, 2007), we undertook a study to determine the prevalence of *Ft* in soil from eight districts of Punjab province of Pakistan followed by an evaluation of seroconversion in small and large ruminants. Besides several soil characteristics that included macro- and micronutrients, the study also examined likely risk factors that could be associated with its occurrence in soil, and therefore can contribute toward human and animal exposures. It is anticipated that the findings will be valuable to local, as well as global public health agencies for

evaluating potential disease burden, identifying reservoirs, and developing strategies to prevent and control tularemia in animal and human populations.

## MATERIALS AND METHODS

### Study and Sampling Design

A cross-sectional study was conducted in Punjab province (31.1704°N and 72.7097°E) from 2011 to 2015. The province has nine administrative divisions, 36 districts and approximate 4,883 villages. It dominates agriculture, and has the largest human and livestock populations in the country. Punjab province contains five rivers (“punj” means five and “ab” means water) which together provide one of the country’s largest irrigation systems for agricultural cultivation. Besides rivers and canals, ground-water (tube-well) as well as natural rain (barani) are being used to irrigate some of the areas in the province. Though mechanical (automatic) plowing is widespread across many districts in the province, animal-based plowing (manual) is also employed at some places in the province. We used three-stage sampling design. Since an incidence rate of *Ft* in Pakistan is not known, we selected districts representing the main livestock production areas of the province where there exists an increased annual incidence of human and animal disease (Directorates of Human and Animal Health, Punjab Province).

Assuming 50% prevalence, 95% CI and 5% margin of error, the required number of villages was 357, however, we included 456 villages representing 10% of each of the study district to increase the validity of the results using WinEpi software (<http://www.winepi.net/uk/sample/indice.htm>). From each village, we conveniently selected five sites for soil sampling; four were from livestock barns where human and animals were living in close proximity, while one represented an agricultural land only. The geographical coordinates were noted using Garmin (Dakota, U.S.A). After removing 3–5 inches of top-surface soil at each site, a total of 2,280 sample (~250–300 gram each) were collected from 456 villages representing districts Sheikhpura ( $n = 295$ ), Gujranwala ( $n = 360$ ), Faisalabad ( $n = 370$ ), Sargodha ( $n = 370$ ), Sahiwal ( $n = 255$ ), D.G. Khan ( $n = 215$ ), Chakwal ( $n = 190$ ), and Attock ( $n = 225$ ). A brief history of each study site along with information about different categorical variables or risk factors such as distance from animal market, main road and water source, animal’s density in a village, number of households in a village, number of domestic animals in a village, cover ground (vegetation) was recorded (Table 1).

### Genome Extraction and PCR Based Identification

Genomic DNA was extracted from 0.25 gram of each soil sample (PowerSoil® DNA Isolation Kit, MoBio, USA) as per manufacturer’s instruction, and was subjected to real time PCR (CFX 96, BioRad, USA) using a highly sensitive and specific assay (Christensen et al., 2006) with minor modifications. The quantification of DNA was performed using the NanoDrop 1000 spectrophotometer (Thermo scientific, USA). The used real time PCR (RT-PCR) primers (Forward:

**TABLE 1** | Eigenvalues and percentage of inertia explained by each principal component.

Component	Initial eigenvalues			Extraction sums of squared loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.143	30.619	30.619	2.143	30.619	30.619
2	1.914	27.339	57.957	1.914	27.339	57.957
3	1.351	19.299	77.256	1.351	19.299	77.256
4	0.895	12.783	90.039			
5	0.348	4.978	95.017			
6	0.285	4.076	99.093			
7	0.064	0.907	100.000			

5'-CAGCATAACAATAAACCCACAAGG-3' and Reverse: 5'-TCAGCATACTTAGTAATTGGGAAGC-3') and probe [5' (FAM)-TTACAAATGGCAGGCTCCAGAAGGTT-3' (TAMRA)], which targeted the *tul4* gene (Lipoprotein/outer-membrane protein, 103 bp). A 25  $\mu$ L reaction was comprised of a final concentration of 1X PCR buffer, 5 mM of MgCl<sub>2</sub>, 0.25 mM of dNTPs, 0.25 mg/mL of bovine serum albumin, 0.6  $\mu$ M of each of forward and reverse primer, 0.025  $\mu$ M of probe and 0.5 U of Taq-polymerase along with soil-extracted genomic DNA (10–30 ng). Thermal cycling conditions included one cycle of 95°C for 5 min followed by 45 cycles of each of denaturation at 94°C for 5 s and annealing at 60°C for 20 s, and then one cycle of cooling at 40°C for 1 min. The assay was optimized and validated using the control (*tul4* gene PCR products) and the proficiency testing samples that were kindly provided by the Pennsylvania State University, USA. The necessary assay controls such as dsDNA PCR product (positive control) and dDiethyl-pyrocyanate water (negative control) were used each time. To rule out any potential contamination in processing and/or false-positivity, each sample that exhibited a positive result was gel-electrophoresed (**Supplementary Material**), and the process described above was repeated thrice beginning from the genome extraction.

## Serum Analysis

Blood samples (~5 mL) were collected conveniently from *Ft*-positive site representing goat ( $n = 200$ ), sheep ( $n = 175$ ), cattle ( $n = 179$ ), and buffalo ( $n = 153$ ) representing district Chakwal, Gujranwala, Faisalabad, Attock, Sahiwal, Sargodha, and Dera Ghazi Khan in Punjab Province, Pakistan. The separated sera were stored at  $-80^{\circ}\text{C}$  until further use. Sera (1  $\mu$ L) were analyzed for anti-*Ft*-enzyme linked immunosorbent assay (ELISA) antibodies using an SERION ELISA classic *Ft* kit (Virion/Serion, Germany) according to the manufacturer's instructions. A specific secondary alkaline phosphatase antibody (ThermoFisher, USA) was used for goat (F (ab')<sub>2</sub>-rabbit anti-goat IgG H+L), sheep (F(ab')<sub>2</sub>-donkey anti-sheep IgG H+L), cattle (goat anti-bovine IgG (H+L)), and buffalo (goat anti-bovine IgG H+L). Optical density of sera were read within 60 min at 405 nm against substrate blank and 655 for reference and field samples as per manufacturer's recommendations. Only valid samples with OD value of substrate blank <0.25 and variation between OD values of standard serums not higher than 20% were

considered for further analysis. The positive and negative serum samples were analyzed using Microsoft<sup>®</sup> Excel-based software tool SERION activity with reference to lower cut off value 0.42 and upper cut off value 1.43 provided by manufacturer. Samples below the lower cut off value were considered as negative while samples above the upper cut off value were measured as positive.

## Soil Chemistry Analysis and Risk Factors

Soil samples (~500 g) were analyzed, using previously optimized protocols for pH (Committee et al., 1978), moisture (McLean, 1982), texture (Robert and Frederick, 1995), total soluble salts (Magistad et al., 1945), phosphorus (Brown, 1998), copper, chromium, calcium, nickel, manganese, iron, cobalt, lead, cadmium, sodium, magnesium, and potassium (Soltanpour and Schwab, 1977), nitrogen (Fierer et al., 2001), and organic matter (Nelson and Sommers, 1982).

## Statistical Analysis

The RT-PCR results along with numerical (soil characteristics) and categorical variables (potential risk factors) were compiled into a Microsoft Excel spreadsheet. In an earlier study (Muhammad et al., 2017), we described association between physio-chemical characteristics of soil and the presence/absence of *Ft* DNA in soil samples. In this study, we further explored these data and other potential variables to quantify factors associated with detection of bacterial DNA in soil. The data on physio-chemical characteristics of soil were not normally distributed (Shapiro-Wilk test,  $p \leq 0.05$ ); therefore, Mann-Whitney *U*-test was applied to assess the effect of those numeric variables on soil positivity. In **Figure 2** the variables with  $p \geq 0.2$  were included in further analyses. The correlation matrix plot revealed collinearity among the selected variables, and correlated variables ( $r \geq 0.3$ ) were subjected to principal component analysis (PCA) (Abdi and Williams, 2010; Pourhoseingholi et al., 2012). PCA reduces dimensionality in the data and transforms the variables into a new set of uncorrelated variables called principal components. The Bartlett test of sphericity was significant ( $p < 0.05$ ) and The Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy was 0.582 ( $p < 0.05$ ), indicating appropriateness of dataset for PCA. The first three principal components had an eigenvalue >1 and were used as covariates in binary logistic regression. A Chi Square test was used to evaluate association between the occurrence of *Ft* DNA in soil and categorical variables. A Fisher

Exact test was used as alternative to Chi square test where any of its assumption was violated. The regression model included the presence/absence of *Ft* DNA in soil samples as a dependent variable. The independent variables in the model were moisture, P, Ni, Mn, Na, Zn, PC1, PC2, and PC3. The model also included categorical variables with  $p \geq 0.2$ . A backward likelihood ratio method was used to select covariates in the regression. A  $p \leq 0.05$  was considered significant in the outcome of regression analysis (Nandi et al., 2016). The data were analyzed in R using “FactoMineR,” “factoextra,” and “corrplot” packages (Table 3).

## RESULTS

### The Prevalence of *Ft* in the Soil of the Studied Districts

Because of differences in the density of the villages in each district, the number of villages and samples varied accordingly from 38 in Chakwal ( $n = 190$ ), to 74 in both Sargodha and Faisalabad ( $n = 370$  each). *Ft* DNA was detected in 74 of 2,280 samples (3.24%, 95% CI: 2.60–4.06). There were some villages where *Ft* DNA was detected at more than one location. These included, one each from Attock (Gharibwal) and Faisalabad districts (482-GB). Compared to the other districts, an increased incidence rate of *Ft* was observed in Faisalabad ( $n = 15$ ), Gujranwala ( $n = 17$ ), and Attock ( $n = 10$ ). The prevalence was highest in district Chakwal [(13.1%, (5.26%, 95% CI: 2.88–9.41)] followed by Gujranwala (4.72%, 95% CI: 2.97–7.43), Attock (4.44%, 95% CI: 2.43–7.98), Faisalabad (4.05%, 95% CI: 2.47–6.58), Dera Ghazi Khan (3.72%, 95% CI: 1.90–7.17), Sargodha (3.24%, 95% CI: 1.86–5.58), and Sahiwal (0.78%, 95% CI: 0.21–2.81). None of the soil sample originating from district Sheikhupura were positive for *Ft* DNA (Figure 1). Interestingly, a total of 20 samples (4.3%, 95% CI: 2.86–6.68) representing agriculture land with no apparent human interaction were also found to be positive where there was no apparent animal and human interaction.

### Relationship Between Soil Chemistry and Categorical Variables to Occurrence of *Ft* in Soil

In the earlier study, we described association between physio-chemical characteristics of soil and presence/absence of *Ft* DNA in soil samples using a simple *t*-test and chi-square (Muhammad et al., 2017). In this study, we further explored these data and other potential variables to quantify factors associated with detection of bacterial DNA in soil. Table 1 shows eigenvalues, percentage variance and cumulative percentage of variance of principal components. The first three principals had eigenvalues  $> 1$  and cumulatively explained 77.26% of total variance (inertia) in the dataset. Varying between  $+ 1$  and  $-1$ , the values ( $\geq 0.4$ ) of the loadings represent the correlation between each variable and a principal component (Table 2). As the absolute value of the loading increases, the importance of the variable to the principal component becomes greater. Component 1 (PC1) explained 30.619% variance. It loaded positively on clay, Cd, Pb and negatively on slit, organic matter, and N. Component 2 (PC2) explained 27.339 % variance. It loaded positively on organic matter and N and negatively on silt. Component 3 (PC3) explained 19.299% variance and loaded positively on silt, Cd, Pb and negatively on clay. The variable *Ft* was significantly linked to PC1 and PC3 ( $p \leq 0.05$ ). For PC1, variable *Ft* explained 26% variance in the coordinates whereas correlation coefficient was 5% for PC3. The PC1 and PC3 have partitioned data into *Ft* positive and negative sites. Figure 3 displays quality of representation (squared cosine,  $\cos^2$ ) of variables and individuals (sampling sites) along the first two principal components. The angles between variables show degree of correlation, and their lengths represent importance for the respective components. Clay, Cd, and Pb were evidenced to be more important for PC1. Organic matter and N were contributed greatly in PC2. Clay and slit were negatively correlated with each other. The sampling points in the periphery had better representation ( $\cos^2$  values closer to 1).

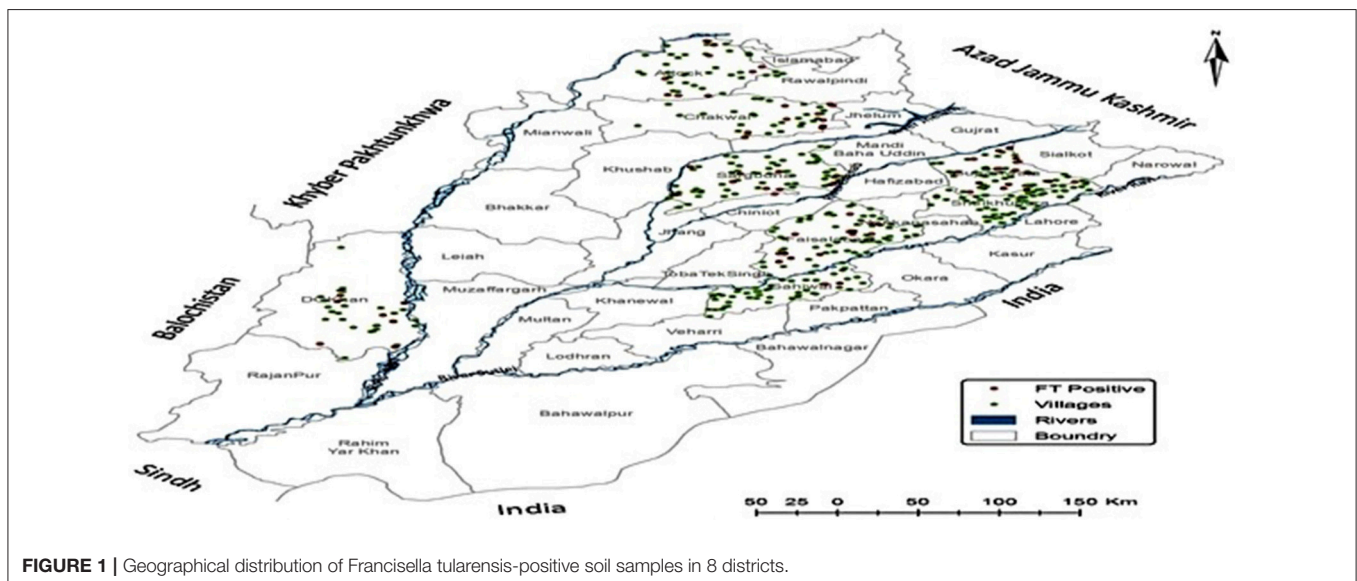


FIGURE 1 | Geographical distribution of Francisella tularensis-positive soil samples in 8 districts.

The logistic regression analysis had AIC value (143.64), Nagelkerke R Square (0.547) and  $-2$  Log likelihood (124.352). The accuracy of the model was 81.4. The Hosmer & Lemeshow test of the goodness of fit suggested fitness of the model at  $p = 0.792$  ( $>0.05$ ). Variables significantly associated with positivity of soil samples were soluble salts (OR: 1.276, 95% CI: 1.043–1.562), Ni (OR: 2.910, 95%CI: 0.795–10.644), Mn (OR:0.733, 95% CI:0.565-0.951), Zn (OR: 4.922, 95% CI:0.929–26.064), and those clustered together as PC1 (OR: 4.760, 95% CI:2.373–9.548) and PC3 (OR:0.357, 95%CI:0.199–0.640), distance from animal market (OR: 0.57, 95% CI:0.30–1.07), distance from water source (OR:1.19, 95%CI: 1.19–3.09), Animal density (OR:1.56, 95%CI: 0.97–2.43), number of households in a village (OR:0.38, 95% CI: 0.20–0.71) and domestic animals in a village (OR:2.069, 95% CI: 1.05–4.05). A unit increase in PC1 means an increase in the risk of soil positivity whereas PC3 had protective effect. Moisture, P, Na, and PC2 were non-significant and therefore removed from the regression equation through

stepwise methods of covariate selection. The experimentation with regression revealed that varimax rotation and dropping some of the correlated variables did not improve the model (AIC, Nagelkerke R Square and  $-2$  Log likelihood values not shown).

### Seroconversion in Domestic Animals to *Ft* in the Studied Districts

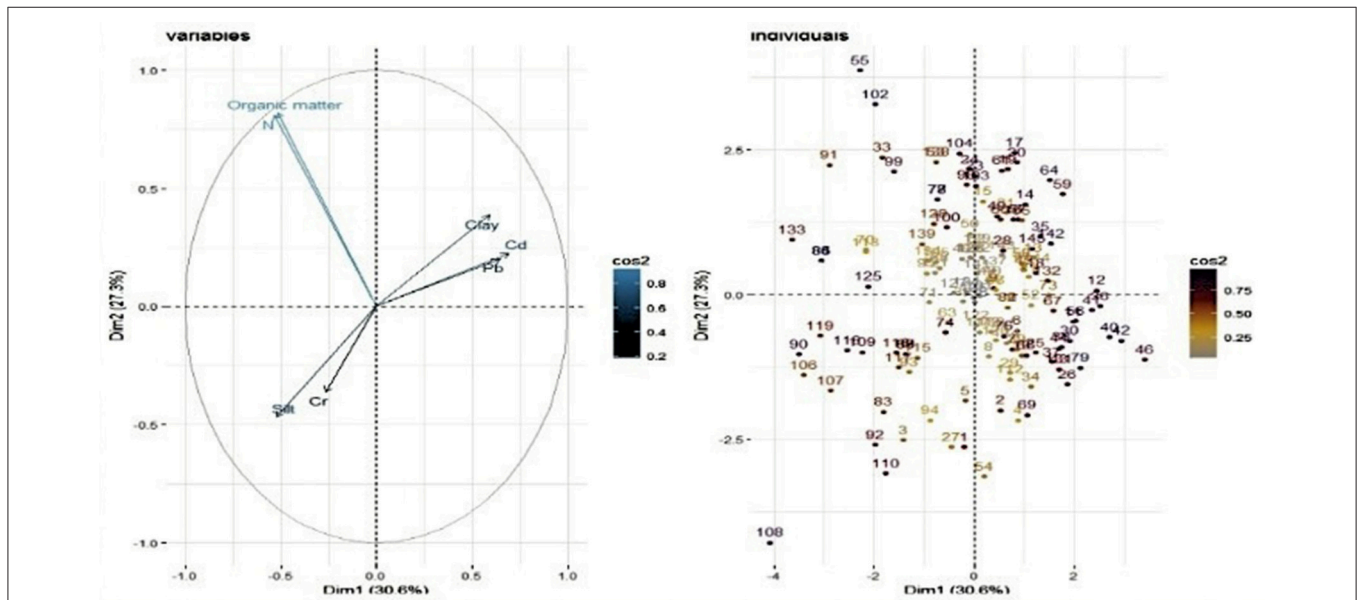
The seroconversion was found in 6.22% of small and large ruminants ( $n = 44$ , 95% CI: 4.67–8.25) Spatial distribution of seropositivity in animals has been illustrated in **Figure 4** where a village has been red highlighted corresponding to seroconversion in any of the study animals. A significant difference ( $p < 0.05$ ) was found in the prevalence of serum anti-*Ft*-ELISA antibodies among cattle (11.17%, 95% CI: 7.35–16.62), and buffalo (8.49%, 95% CI: 5.04–14.0), sheep (5.7%, 95%CI: 3.13–10.19), and goat (0.5%, 95% CI: 0.00–2.78). The exposure rate was much more in large ruminants (9.94%, 95% CI: 7.17–13.63) than small ruminants (2.93%, 95 CI: 1.64–5.17). Likewise, a significant difference was observed for gender ( $\chi^2 = 15.35$ ,  $p = 0.000$ ) where seropositivity was much more in female animals (16.3%) than male animals (6.2%) while a non-significant difference was observed in age groups of small [ $<2$  years (4%) v/s 2–4 years (2%) v/s  $>4$  years (14.2%),  $\chi^2 = 3.81$ ,  $p = 0.14$ ] and large ruminants [ $<3$  years (10.8%) v/s 3–6 years (7.9%) v/s  $>6$  years (33%),  $\chi^2 = 1.85$   $p = 0.39$ ].

**TABLE 2 |** Factor-loading matrix for the phyto-chemical attributes of soil sampled identified by principal component analysis.

	Component		
	1	2	3
Silt	-0.524	-0.467	0.572
Clay	0.593		-0.567
Organic Matter	-0.515	0.820	
Cd	0.693		0.523
Cr			
Pb	0.646		0.623
N	-0.533	0.809	

### DISCUSSION

The molecular diagnostic assay (RT-PCR) had a high sensitivity and specificity with a detection limit as low as  $<100$  genome copies, and therefore allows simultaneous examination of numerous samples with rapid turnaround time (Christensen



**FIGURE 2 |** Biplot showing representation of sampling points (called individuals) and physiochemical attributes (variables) in multivariate principal component space.



**TABLE 3 |** Outcome of logistic regression analysis showing association between presences of DNA of *Francisella tularensis* soil samples and its potential risk factors.

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I. for EXP(B)	
							Lower	Upper
Soluble salts	0.244	0.103	5.606	1	0.018	1.276	1.043	1.562
Ni	1.068	0.662	2.606	1	0.106	2.910	0.795	10.644
Mn	-0.310	0.133	5.483	1	0.019	0.733	0.565	0.951
Zn	1.594	0.850	3.511	1	0.061	4.922	0.929	26.064
PC1	1.560	0.355	19.294	1	0.000	4.760	2.373	9.548
PC3	-1.030	0.298	11.934	1	0.001	0.357	0.199	0.640
Distance from animal market	0.534	0.975	9.318	1	0.05	0.57	0.30	1.07
Water source (canal/stream/drain)	-2.051	2.762	13.956	1	0.004	1.19	1.19	3.09
Animal density in a village	0.927	0.175	8.59	1	0.07	1.56	0.97	2.43
Number of households in a village	-1.823	0.690	3.489	1	0.002	0.38	0.20	0.71
Domestic animal	0.121	0.378	7.321	1	0.02	2.069	1.05	4.05
Ground cover (vegetation)	-0.781	0.782	6.812	1	0.08	1.49	0.88	2.51
Constant	-3.647	1.381	6.978	1	0.008	0.026		

2014). Previous studies have shown that *Ft* can survive in water, soil, mud, animal waste, and frozen meat for an extended period of time (Eliasson et al., 2006; Sjöstedt, 2007). An area in close proximity to water may have a higher probability for the presence of *Ft*, however the presence of mammalian host may be required for manifestation of disease (Desvars et al., 2015). Although an increased persistence of *Ft* has been observed in environments lacking humidity (Wilkinson, 1966; Dennis et al., 2001), the organism cannot remain viable under desiccation conditions (Faith et al., 2012). A significant association between the occurrence of *Ft* in soil and the presence of animals at the same site may correspond to the presence of disease reservoirs at locations in Punjab province where *Ft* was identified, such as the rodents that are found frequently at livestock barns and surrounding areas. It has been observed that an increase in the human population can decrease the number of rodents in the fields and corresponding areas (Sutherst, 2004); in contrast, an increase in the animal population can lead to an increase in the rodent population and consequently an increase in the risk of *Ft* (Gürçan, 2014). Most of the positive samples were collected in irrigated and cropland areas (Chakwal, Gujranwala, and Faisalabad), that are interconnected with each other. In fact, compared to areas with forests and water, a previous study showed that *Ft* was primarily observed in croplands where rodents were found to be a common vector for *Ft* infections (Hightower et al., 2014).

Our findings show that it is difficult to define the relationship between the characteristics of the studied analytes and the persistence of *Ft* in soil. However, this study provides insight between *Ft* and its relationship with soil texture and metals requiring that further investigations are conducted in order to ascertain more definitive outcomes. Metals including iron, zinc, copper, and manganese play a key role in biochemical reactions and are present in most of the organism as constituents of different biological molecules (Hood and Skaar, 2012). Copper, magnesium, manganese, zinc, nickel, cadmium and sodium were

observed to have a role in persistence of *Ft* in soil, although further investigation of these elements is needed. Indeed, some studies emphasized that survival and persistence of different organisms in soil is influenced by the soil texture (Nicholson et al., 2005). For instance, *Burkholderia pseudomallei* and *Ft* has the potential to survive for 30 months and 14 weeks, respectively, in clay soil (Thomas and Forbes-Faulkner, 1981; Cieslik et al., 2018). Generally, soil with an increase in clay support enhanced growth of organisms as compared to soil with increased concentration of sand (Locatelli et al., 2013). A potential association between *Ft* DNA and Magnesium could be correlated with its role while maintaining negative charge in LPS of outer membrane and therefore stability of organism in unfavorable conditions (Leadbetter and Poindexter, 2013; Wu et al., 2016). Similar to *Ft*, intracellular bacteria including *Salmonella enterica*, *Listeria monocytogenes*, *Brucella abortus*, and *Yersinia pestis* require zinc for intracellular survival and replication (Desrosiers et al., 2010; Corbett et al., 2012; Ma et al., 2015). Cadmium is normally toxic to bacteria however it has several mechanisms to develop resistance including (1) enzymes which make cell wall impermeable, (2) mechanism of efflux, (3) binding of metal ions, and (4) enzymes which convert toxic metals to non-toxic (Ron et al., 1992; Abbas et al., 2017). The persistence of *Ft* in cadmium high concentration soil presents a potential mechanism of resistance that need to be explored in future studies.

A large proportion of ruminants (goat, sheep, cattle, and buffalo) were found to be seropositive for anti-*Ft* antibodies. All the seropositive animals were clinically healthy and were devoid of symptoms suggestive of *Ft* infection. This is not surprising, because both active and passive forms of *Ft* have previously been evidenced in humans, rodents, and wild animals, (Wobeser et al., 2009) and therefore a varying rate (4–19%) of asymptomatic tularemia could be expected (Kiliç, 2010). Indeed, the clinical form of the infection depends on the route of entry of the pathogen into the body and the immune status of the host

(Gürçan, 2014). The percent prevalence of anti-*Ft* antibodies was significantly higher in large ruminants (cattle and buffalo) than small ruminants (sheep and goat). Interestingly, previous studies have shown that cattle and sheep are comparatively resistant to *Ft* and remain asymptomatic (Mörner et al., 1988; Pfahler-Jung, 1989; Winn and Koneman, 2006). Some animals are very sensitive to tularemia, and succumb to infection soon after development of specific antibodies, whereas cattle, pigs, and sheep are more resistant, despite even having a significantly high antibody titer (Hopla, 1974; Valdes and Valdes, 2018). Animals may not show clinical signs even after years of infection, possibly owing to the presence of post-exposure cellular and humoral immunity (Bevanger et al., 1994; Ericsson et al., 1994; Magnarelli et al., 2007). A recent study revealed that both small and large ruminants, independent of their genders, are frequently exposed to soil contaminated with *Ft* supporting the findings by previous studies showing that cattle maintain some degree of *Ft* resistance (Dienst Jr, 1963; Scheel et al., 1992; Jacobs, 2002). Similarly, another study showed that both male and female mice are susceptible to *Ft* where male mice develop severe clinical signs and high mortality as compared to female (Sunagar et al., 2016). These observations may be due to differences in immune response development in male and female mice. An example of this was shown during *Streptococcus pneumoniae* infections, where immune system inefficiency was noted to be higher in female mice as compared to male mice (Wiemken et al., 2014).

In conclusion, this study provides a preliminary confirmation of the presence of *Ft* in the Punjab Province of Pakistan and its potential association with several soil characteristics (macro- and micro-nutrients) at a higher statistical resolution. Future studies involving unexplored geographical areas of the country, disease reservoirs including rodents, and evaluation of the at-risk human population (the farmers and veterinarians), are needed.

## ETHICS STATEMENT

Blood samples were collected from cattle, buffalo, sheep, and goat following the guidelines of International Animal Care and Use Committee (IACUC) with prior consent of the farm's owner. All samples were analyzed after approval from the Ethical

Research Board at the University of Veterinary and Animal Sciences, Lahore, Pakistan vide approval No: DR/236 dated 16th May, 2013.

## AUTHOR CONTRIBUTIONS

BMJ, MZS, MR and KM: conceived and designed the study; JM, MZS, MH, ZUH, HRC, MTG, TJ, and MAA: sample collection and laboratory procedure across the study districts; JM, MZS, MN, TA and MHC: data analysis; JM, MZS, GSK and BMJ: manuscript write-up and necessary editing. All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2019.00089/full#supplementary-material>

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