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Institut für Bakterielle Infektionen und Zoonosen, Jena

eingereicht über das
Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Diagnosis and molecular biology of Brucella abortus in Pakistan

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
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vorgelegt von
Tariq Jamil
Tierarzt aus Bahawalpur, Pakistan

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List of publications in peer reviewed journals

1. **Jamil, T.**, Melzer, F., Njeru, J., El-Adawy, H., Neubauer, H., Wareth, G. (2017). *Brucella abortus*: current research and future trends. Current Clinical Microbiology Reports. 4(1): 1-10. http://dx.doi.org/10.1007/s40588-017-0052-z.
2. **Jamil, T.**, Melzer, F., Saqib, M., Shahzad, A., Kasi, K.K., Hussain, M.H., Rashid, I., Tahir, U., Khan, I., Tayyab, M.H., Ullah, S., Mohsin, M., Mansoor, M.K., Schwarz, S., Neubauer, N. (2020). Serological and molecular detection of bovine brucellosis at institutional livestock farms in Punjab, Pakistan. International Journal of Environmental Research and Public Health. 17(4): 1412. https://doi.org/10.3390/ijerph17041412.
3. **Jamil, T.**, Melzer, F., Khan, I., Iqbal, M., Saqib, M., Hussain, M.H., Schwarz, S., Neubauer, H., 2019. Serological and molecular investigation of *Brucella* species in dogs in Pakistan. Pathogens. 8(4): 294. https://doi.org/10.3390/pathogens8040294.
4. Ullah, Q., **Jamil, T.**, Melzer, F., Saqib, M., Hussain, M.H., Jamil, H., Iqbal, M.A., Tahir, U., Ullah, S., Qureshi, Z.I., Schwarz, S., Neubauer H., (2020). Epidemiology and associated risk factors for brucellosis in small ruminants kept at institutional livestock farms in Punjab, Pakistan. Frontiers in Veterinary Science. 7: 526. doi: 10.3389/fvets.2020.00526 .
5. **Jamil, T.**, Melzer, F., Kasi, K.K., Saqib, M. Ullah, Q., Khan, M.R., Tayyab, M.H., Schwarz, S., Neubauer, H. (2020). Revisiting brucellosis and associated risk factors in small ruminants of Western border areas in Pakistan. Veterinary Medicine and Science. (**Under review**).
6. Ali, S., Neubauer, H., Melzer, F., Khan, I., Akhter, S., **Jamil, T.**, Umar, S., (2017). Molecular identification of bovine brucellosis causing organisms at selected private farms in Pothohar Plateau, Pakistan. Pakistan Journal of Zoology. 49 (3): 1111-1114. doi: 10.17582/journal.pjz/2017.49.3.sc2.
7. Ullah, Q., Jamil, H., Lodhi, L.A., Qureshi, Z.I., Ullah, S., **Jamil, T.**, Khan, I., Bashir, S., Qudratullah, Wazir, I., Sallam, M.A., Zubair, M. (2019). Brucellosis is significantly associated with reproductive disorders in dairy cattle of Punjab, Pakistan. Pakistan Journal of Zoology. 51(5): 1995-1997. doi:10.17582/journal.pjz/2019.51.5.sc10.

The publications 1-4 are part of this dissertation.

List of publications in academic conferences/symposia

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1. **Jamil, T**., Ullah, Q., Melzer, F., Saqib, M., Hussain, M.H., Tahir, U., Neubauer, H., Schwarz, S. Seroprevalence and molecular detection of small ruminant brucellosis in Pakistan. 8th Leipziger Doktorandenforum: Oral presentation, 13 Feb 2020, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany.
2. **Jamil, T**., Melzer, F., Saqib, M., El-Adawy, H., Neubauer, H., Schwarz, S. Update of bovine brucellosis at livestock farms in Punjab, Pakistan. Junior Scientist Symposium 8 (Jena): Oral presentations: Epidemics, p.25. 25-27 Sep 2019, Friedrich-Loeffler-Institut, Jena, Germany.
3. **Jamil, T.**, Kasi, K.K., Melzer, F., Neubauer, H., Schwarz, S. Brucellosis in small ruminants in three divisions in Baluchistan, Pakistan. Oral presentation, 11th Doktorandensymposium & DRS Präsentationsseminar „Biomedical Sciences“, 21 Sep 2018, Faculty of Veterinary Medicine, Freie Universität, Berlin, Germany **(Won second prize for best talk).**
4. **Jamil, T.**, Kasi, K.K., Melzer, F., El-Adawy, H., Khan, I., Elschner, M., Neubauer, H., Schwarz, S. Brucellosis in small ruminants in three divisions in Baluchistan, Pakistan. One Health International Conference (OHC-2017): Oral presentation, 13-15 Nov 2017, University of Veterinary & Animal Sciences (UVAS), Lahore, Pakistan.
5. **Jamil, T.**, Melzer, F., El-Adawy, H., Rabbani, M., Khan, I., Neubauer, H., Schwarz, S. Seroprevalence and molecular detection of *Brucella* in stray dogs in Pakistan. Junior Scientist Symposium 6 (Braunschweig): Oral presentation: p.21. 20-22 Sep 2017, Friedrich-Loeffler-Institut, Braunschweig, Germany.

Poster presentations:

1. Ullah, Q., **Jamil, T**., Melzer, F., Saqib, M., Hussain, M.H., Jamil, H., Iqbal, M.A., Tahir, U., Ullah, S., Qureshi, Z.I., Schwarz, S. Neubauer, H., Seroprevalence and molecular detection of small ruminant brucellosis in Pakistan. 6th Joint Conference of the DGHM & VAAM: 08-11 March 2020, University of Leipzig, Leipzig, Germany.
2. **Jamil, T.**, Melzer, F., Saqib, M., Ullah, Q.; Khan, R.; Tayyab, M. H.; Neubauer, H. An update of brucellosis in the western nomadic ruminants in Pakistan. Junior Scientist Zoonoses Meeting (Berlin): 20-22 June 2019, Berlin, Germany.
3. **Jamil, T.**, Melzer, F; Zubair Shabbir, M.; Ahmad, A; Khan, I; Neubauer, H; Schwarz, S. Zoonotic brucellosis in Pakistan. Junior Scientist Symposium 7 (Greifswald-Insel Riems): Poster presentations p.69. 24-26 Sep 2018, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany.

List of abbreviations

B.	*Brucella*
CAT	Cord Agglutination Test
CFT	Complement Fixation Test
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked Immune sorbent Assay
FLI	Friedrich-Loeffler-Institut
GDP	Gross Domestic Product
i-ELISA	indirect-Enzyme Linked Immunosorbent Assay
c-ELISA	competition-Enzyme Linked Immunosorbent Assay
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry
MLSA	Multi-locus Sequence Analysis
MLST	Multi-locus Sequence Typing
MLVA	Multiple Loci VNTR Analysis
MRT	Milk Ring Test
OIE	Office International des Epizooties
RBPT	Rose Bengal Plate Test
RFM	Retention of Fetal Membranes
SAT	Slow/Serum Agglutination Test
SATT	Serum Agglutination Tube Test
SNP	Single-Nucleotide Polymorphism
SPAT	Standard/Serum Plate Agglutination Test
SQAT	Semi-Quantitative Agglutination Test
STAT	Serum Tube Agglutination Test
USD	United States Dollar
VNTR	Variable Number Tandem Repeat
VRI	Veterinary Research Institute
WHO	World Health Organization

1 Introduction

Brucellosis is a bacterial zoonosis in animals. It is caused by bacteria of the genus *Brucella* (*B.*), which are Gram-negative, non-motile, non-spore forming, non-haemolytic and intracellularly living. Brucellosis is an abortive disease and accompanied by fever, retention of fetal membranes in animals, loss of milk production and fertility. Depending upon host preference, *B. abortus* causes infections in bovines and wild ruminants, *B. melitensis* in small ruminants, *B. canis* in dogs, *B. suis* in pigs and *B. ovis* in rams although cross-species transmission is possible via close contact with infected animals (Jamil et al., 2019; Saeed et al., 2019; Saleem et al., 2019). Brucellosis is found worldwide, especially in developing and tropical countries whereas North and Central Europe, Australia, New Zealand, Japan and Canada are considered free of *B. abortus* and *B. melitensis* in domestic animals (Aparicio, 2013). Brucellosis is characterized by abortion in the last trimester and retention of fetal membranes whilst orchitis and epididymitis in males results in overall infertility. The infection can be asymptomatic. Hence, the infected animal may stay undiagnosed. Animals may carry subsequent parturitions, shed bacteria through vaginal and milk secretions in the environment and may transmit the infection to their progeny (Akhtar and Mirza, 1995; Bercovich, 1998; Hull et al., 2018). Bursitis and hygroma of the limbs are also occasional symptoms in animals (Hull et al., 2018; Ocholi et al., 2004).

Brucellosis causes economic losses in terms of abortion, week new-born animals, screening and culling of animals, impediment in trade and milk loss especially affecting progressive farmers with exotic dairy animals raised in developing countries. Brucellosis is usually transmitted by direct contact with infected animals or through ingestion of contaminated feed or water. In humans, it is mainly transmitted via ingestion of contaminated dairy food e.g. milk (Abedi et al., 2020; Dadar et al., 2019; Gul and Khan, 2007). In humans, brucellosis is caused by *B. abortus, B. melitensis, B. suis* and *B. canis* which cause unspecific signs e.g. fever and abortion, which can be misdiagnosed with typhoid, rheumatic fever and other seasonal illnesses (Njeru et al., 2016a; Njeru et al., 2016b). Brucellosis is a public health threat in developing countries where livestock farmers, veterinarians, abattoir workers and butchers are at occupational risk of the infection (Ali et al., 2013; Asif et al., 2014; Mukhtar and Kokab, 2008). Humans are accidental hosts and transmission is prevented by eliminating the infection in animals often having close contact with humans (Rubach et al., 2013). No safe vaccines for humans exist and treatment is often associated with adverse effects and relapses (Lalsiamthara and Lee, 2017). Diagnosis is a challenge and depends upon a combination of clinical history, symptomology, laboratory-based examination of biological specimens (e.g. serum and milk), and the epidemic situation of the disease in the respective geographical area. Conventional tests for *B. abortus, B. melitensis* and *B. suis* depend on smooth-

lipopolysaccharide (S-LPS) antigen which is not produced by *B. canis* and *B. ovis* as they have rough-lipopolysaccharide (R-LPS). Routine serological examinations include Rose Bengal Plate Test (RBPT), Enzyme Linked Immunosorbent Assay (ELISA), Serum Agglutination Test (SAT), Complement Fixation Test (CFT) and Milk Ring Test (MRT). Molecular tests used are e.g. Polymerase Chain Reactions (PCR) or whole genome sequencing (WGS). Culture of the bacteria remains the gold standard but is hazardous and restricted to specialized laboratories (Biosafety Level-III). Thus, diagnosis still relies mainly on serology. Treatment of farm animals is forbidden in many countries and eradication programs finally rely on test and slaughter/culling policy for eradication. However, this is relatively difficult to implement in developing countries due to the higher costs of high-performance animals.

Aims of this study

The aims of this doctoral thesis were to:

1. update the existing knowledge of brucellosis in general, and in specific for the situation in Pakistan,
2. identify the prevailing brucellosis aetiology by serological and molecular biological diagnostic methods in ruminant and non-ruminant domestic animals,
3. study potential risk factors for transmission of brucellosis in these animals, and
4. identify possible solutions for the problems based on the obtained results and develop recommendations.

2 Literature overview

The review paper "*Brucella abortus*: Current research and future trends" published by Jamil, T., Melzer, F., Njeru, J., El-Adawy, H., Neubauer, H., Wareth, G. in *Current Clinical Microbiology Reports* (2017) 4:1-10. https://doi.org/10.1007/s40588-017-0052-z served as literature overview of this thesis.

3 Publications

3.1 Publication 1

Jamil, T., Melzer, F., Saqib, M., Shahzad, A., Kasi, K.K., Hussain, M.H., Rashid, I., Tahir, U., Khan, I., Tayyab, M.H., Ullah, S., Mohsin, M., Mansoor, M.K., Schwarz, S., Neubauer, N. (2020). Serological and molecular detection of bovine brucellosis at institutional livestock farms in Punjab, Pakistan.

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Article

Serological and Molecular Detection of Bovine Brucellosis at Institutional Livestock Farms in Punjab, Pakistan

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Abstract: Bovine brucellosis remains a persistent infection in ruminants in Pakistan. A total of 828 (409 buffaloes and 419 cattle) sera were collected from 11 institutional-owned livestock farms in Punjab, Pakistan. The samples were tested by rose bengal plate agglutination test (RBPT) and indirect enzyme-linked immunosorbent assay (iELISA). The seroprevalence along with 95% confidence interval (CI) was determined. Univariable and multivariable analysis of the epidemiological background data was conducted and odds ratio (OR) was calculated to understand any association between the risk factors and the seroprevalence. An overall seroprevalence of 3.9% (Positive/Tested = 32/828) and 3.3% (27/828) was detected by RBPT and iELISA, respectively. The seroprevalence of 5.6% (CI 3.6–8.3) and 4.7%, (CI 2.8–7.2) and the odds ratio of 2.63 (CI 1.20–5.77) and 2.50 (CI 1.08–5.78) for testing positive by RBPT and iELISA, respectively were significantly higher ($p < 0.05$) in buffaloes than in cattle. Breed, sex, history of abortion and retention of fetal membranes (RFM) in the animals were not found statistically significantly associated with the infection. RBPT and iELISA based results agreed almost perfect ($k = 0.877$). In total, *Brucella abortus*-DNA (9/27) was amplified from seropositive samples by real-time polymerase chain reaction. This study identified for the first time the etiological agents of brucellosis at a molecular level at institutional-owned livestock farms in Pakistan.

Keywords: bovine brucellosis; zoonosis; *Brucella abortus*; Pakistan

1. Introduction

Brucellosis is a bacterial zoonosis caused by bacteria of the genus *Brucella* (*B.*). They are non-spore forming, non-motile, non-hemolytic and facultative intra-cellular living, Gram-negative coccobacilli. Although Brucellae show a certain host preference, e.g., *B. abortus* prefers bovines and *B. melitensis* small ruminants, cross-species transmission does occur when different animals are in close contact with each other [1–6]. Brucellosis occurs worldwide, especially in developing and tropical countries, whereas North and Central Europe, Australia, New Zealand, Japan, and Canada are considered as being free from conventional brucellosis in domestic animals [7]. Abortion in the last trimester and retention of fetal membranes (RFM) are the characteristic signs in female animals whilst orchitis and epididymitis commonly occur in males however, the infection may stay asymptomatic and the infected animals may remain undiagnosed [8]. Infected animals shed the bacteria through vaginal and milk secretions in the environment [9]. Brucellosis is usually transmitted in animals either by direct contact or through ingestion of contaminated feed or water whereas in humans, it mainly occurs through ingestion of contaminated milk [10,11]. Humans are accidental hosts for this infection and could be prevented by eliminating the infection in animals that often have close contact with humans [12,13].

The diagnostic confirmation depends on the clinical history, laboratory-based examination of biological specimens, e.g., serum and milk and upon the situation of the disease in the area. The serological examination includes rose bengal plate test (RBPT), enzyme-linked immunosorbent assay (ELISA), serum agglutination test (SAT), complement fixation test (CFT) and milk ring test (MRT) followed by molecular biological investigation, e.g., polymerase chain reaction (PCR), isolation, biochemical identification and molecular typing e.g., multilocus sequence typing (MLST), single nucleotide polymorphism (SNP) and multiple locus variable number tandem repeat analysis (MLVA) etc. [14,15]. Vaccination and treatment of brucellosis in farm animals are not considered 100% safe for human health, hence are forbidden in many countries [7,16–19].

Pakistan is an agriculture-based country where livestock plays an integral role in the agriculture economy. More than 8.0 million families are associated with livestock raising and derive ≥35% of their income from livestock production in the country [20]. Brucellosis is considered an endemic infection in the ruminants in Pakistan [21]. Bovines are the primary source of milk in the country, and for milk production, Pakistan has been among the top countries in the world [22]. Our aim for this study was to estimate the burden of brucellosis in buffaloes and cattle reared at 11 institutional-owned livestock farms by serology and detect the etiology by molecular biology. To the best of our knowledge, this is the first study to identify brucellosis at molecular level at these farms in Pakistan.

2. Materials and Methods

For this study, 11 institutional livestock farms (Farms A–K), administered by the Livestock and Dairy Development (L&DD), Government of Punjab, Lahore and University of Agriculture (UAF), Faisalabad, representing different geographical locations (Figure 1) were selected as described previously [23,24]. Since the prevalence of brucellosis was considered unknown at these selected farms, the sampling frame was constructed to investigate brucellosis at expected prevalence of 50%, 95% confidence interval (CI) and 5% desired absolute precision [25]. This required that at least 385 samples from buffaloes and cattle each to be tested from the selected farms. This sample size was further divided according to the population proportion of these animals at each farm. A total of 828 sera (409 buffalo and 419 cattle) were sampled. Animals were randomly selected and properly restrained before the blood was drawn into a 9-mL vacutainer tube by puncturing the jugular vein. Samples were labelled with the animal identification information (tag number, age, breed, and sex). Epidemiological information regarding the animal and herd level variables were recorded on a questionnaire. The samples were then transported to the Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan where serum was separated and stored at −20 °C until further testing.

Sera were screened for brucellosis by RBPT using Pourquier® Rose Bengal Antigen (IDEXX, Montpellier, France) by using bovine bacterial positive and negative control sera provided by Friedrich-Loeffler-Institut (FLI), Jena, Germany. It was followed by indirect-Enzyme Linked Immunosorbent Assay (iELISA) via ID Screen® Brucellosis Serum Indirect Multi-species (IDVet, Grabels, France) for detection of anti-smooth-Lipopolysaccharide (LPS) antibodies (*B. abortus*, *B. melitensis* and *B. suis*) as per manufacturer's recommendations. The sera were then subjected to DNA extraction by Blood Genomic DNA Extraction Mini Kit (Favorgen®, Ping-Tung, Taiwan) followed by detection/differentiation of Brucellae at species level by real-time PCR using SYBR® Green as described earlier by using previously described sets of primers [26,27]. Each DNA extraction procedure was run along with *E. coli* negative controls and *B. abortus* (Veterinary Research Institute, Lahore, Pakistan) and *B. melitensis* (University of Agriculture, Faisalabad, Pakistan) [6] were used as positive controls in PCR procedure. As no reports on *B. suis* were available in the country, we considered *B. suis* was not prevalent in the area, hence no controls were used. Based on our in-house experience, a cycle threshold (Ct) value of ≤35 was considered as positive [27].

Statistical Analysis

The statistical analysis was conducted by using the R and R-Studio software (RStudio Inc., Boston, MA, USA) [28], and maps were built using ArcGIS version 10.5.1 (ESRI, Redlands, CA, USA). The confidence interval (CI) for the proportions was estimated by the exact 95% Clopper and Pearson interval method using the binom package (binom.test function). Univariate and multivariate analysis were conducted to determine the association and risk (Odds ratio; OR) of the biologically plausible factors with the prevalence of brucellosis. The confirmation of brucellosis was considered as an outcome or dependent variable while possible risk factors were considered as explanatory or independent variables. For the independent variables, biologically plausible variables were considered. The $p < 0.05$ was considered as a level of significance. The Nagelkerke R^2 (NR^2) and Hosmer and Lemeshow Test (HLT) were used to evaluate the final-model fitness. An inter-rater reliability analysis using the Kappa statistics was performed to determine the agreement among two tests, i.e., RBPT and iELISA.

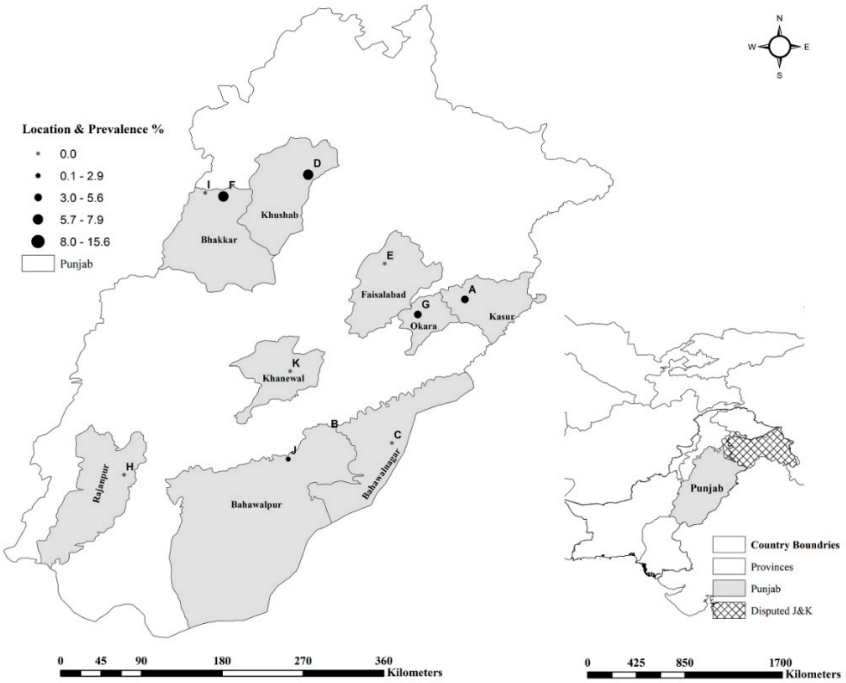


Figure 1. Geographic distribution of brucellosis infection among livestock farms in Punjab, Pakistan.

3. Results

An overall 3.9% (Positive/Tested = 32/828) and 3.3% (27/828) seroprevalence was found by RBPT and iELISA, respectively, among the livestock farms sampled in Punjab, Pakistan (Tables 1 and 2).

For risk factor variables, the sampled animal population (n = 828) was divided into two categories, i.e., buffalo (n = 409) and cattle (n = 419). For the breed variable, two groups were categorized, i.e., local bred animals encompassing Nili-Ravi (n = 409) in buffaloes (n = 409) and Sahiwal (n = 335), Cholistani (n = 46) and crossbred (n = 38) in cattle. Based on sex, animals were grouped into buffalo males (n = 6) and females (n = 403) and cattle males (n = 43) and females (n = 376). Age groups, i.e., <2 years comprised young stock in buffaloes (n = 77) and cattle (n = 95) and ≥2 years comprised bulls, heifers, pregnant and lactating animals in buffaloes (n = 332) and cattle (n = 324). Although retention of fetal membranes (RFM) and history of abortion are purely related to females and prior pregnancy status, males and heifers were considered animals being negative for prior history for RFM and abortion. All sampled animals (n = 828) had no prior history of vaccination against brucellosis at these farms. At the time of sampling, the 11 farms had either only buffaloes (n = 4), only cattle (n = 4) or both, buffaloes and cattle (n = 3) (Table 1).

Species wise in buffaloes, the mean seroprevalence was 5.62% (23/409; range 0–18.75%) by RBPT and 4.64% (19/409; range 0–15.62%) by iELISA at the sampled farms. The highest seroprevalence was found at Farm B with gradual decrease to 0% at Farm C and Farm E respectively, by both tests. Similarly in cattle, the mean seroprevalence was 2.15% (9/419; range 0–6.3%) by RBPT and 1.91% (8/419; range 0–5.52%) by iELISA with highest at Farm G decreasing to 0% at Farms E, F, H, I and K by both tests. The seroprevalence varied statistically significant ($p < 0.05$) by both RBPT (Chi-square value; $\chi^2 = 6.729$) and iELISA ($\chi^2 = 4.69$) between buffaloes and cattle at eleven farms (Table 1). The mean RBPT-based seroprevalence (3.9%) varied (0–18.8%) statistically significant ($\chi^2 = 39.680$, $p < 0.05$) among the sampled livestock farms. A similar pattern was found for the iELISA-based seroprevalence (3.3%) varying (0–15.6%) statistically significant ($\chi^2 = 33.498$, $p < 0.05$) among the sampled farms (Table 2).

In univariate analysis, farm-related variables e.g., feeding methods, herd type, breeding methods and farm environment did not show statistically significant associations ($p > 0.05$) to the seropositivity for brucellosis in both buffaloes and cattle. In animal related variables, species of the animals (buffalo or cattle) did show statistically significant association ($p < 0.05$) with odds ratio of 2.7 (1.24–5.94; 95% CI) in buffalo with reference to cattle. Breed of the animal (local breed or cross-breed) and sex of the animal (male or female) could not be determined whereas, age groups (<2 years and ≥2 years), tick infestation, RFM and history of abortion were not found statistically significantly associated. However, age grouping showed a closer value to the significance level (Table 3). Multivariate analysis for species differences showed a statistically significant association ($p < 0.05$) with an Odds ratio of 2.63 (1.20–5.77; 95% CI) in buffaloes as compared to the cattle. Age group difference did not show a statistically significant association, however, and was found closer ($p = 0.065$) to the level of significance (Table 4).

Table 1. Seroprevalence in cattle and buffaloes sampled from various farms.

Sr. No.	Farm Name	Buffalo				Cattle				Real-Time PCR (SYBR® Green)		
		RBPT		iELISA		RBPT		iELISA				
		Pos./Tested	Prev.% (95% CI)	Pos./Tested	Prev.% (95% CI)	Pos./Tested	Prev.% (95% CI)	Pos./Tested	Prev.% (95% CI)	Buffalo	Cow	Total
1	Farm A	3/70	4.3 (0.9–12)	2/70	2.9 (0.3–9.9)	-	-	-	-	0	0	0
2	Farm B	6/32	18.8 (7.2–36.4)	5/32	15.6 (5.3–32.8)	-	-	-	-	3	0	3
3	Farm C	0/35	0 (0–10)	0/35	0 (0–10)	-	-	-	-	0	0	0
4	Farm D	6/76	7.9 (3–16.4)	6/76	7.9 (3–16.4)	-	-	-	-	2	0	2
5	Farm E	0/58	0 (0–6.2)	0/58	0 (0–6.2)	0/45	0 (0–7.9)	0/45	0 (0–7.9)	0	0	0
6	Farm F	7/71	9.9 (4.1–19.3)	5/71	7 (2.3–15.7)	0/19	0 (0–17.6)	0/19	0 (0–17.6)	1	0	1
7	Farm G	1/67	1.5 (0–8)	1/67	1.5 (0–8)	8/127	6.3 (2.8–12)	7/127	5.5 (2.2–11)	0	3	3
8	Farm H	-	-	-	-	0/23	0 (0–14.8)	0/23	0 (0–14.8)	0	0	0
9	Farm I	-	-	-	-	0/75	0 (0–4.8)	0/75	0 (0–4.8)	0	0	0
10	Farm J	-	-	-	-	1/46	2.2 (0.1–11.5)	1/46	2.2 (0.1–11.5)	0	0	0
11	Farm K	-	-	-	-	0/84	0 (0–4.3)	0/84	0 (0–4.3)	0	0	0
	Total	**23/409**	**5.6 (3.6–8.3)**	**19/409**	**4.7 (2.8–7.2)**	**9/419**	**2.2 (1–4)**	**8/419**	**1.9 (0.8–3.7)**	**6/19**	**3/8**	**9/27**

Sr. No.—Serial number; RBPT—Rose Bengal Plate Agglutination Test; iELISA—Indirect Enzyme-Linked Immunosorbent Assay; PCR—Polymerase Chain Reaction; Pos.—Positive; Prev.—Prevalence; CI—Confidence interval; RBPT-based seroprevalence varied significantly between cattle and buffaloes, $\chi^2 = 6.729$, $p = 0.009$. iELISA-based seroprevalence varied significantly between cattle and buffaloes, $\chi^2 = 4.690$, $p = 0.030$.

Table 2. Overall Seroprevalence of brucellosis in cattle and buffaloes sampled from different farms.

Farm Name	RBPT Overall		iELISA Overall	
	Pos./Tested	Prev.% (95% CI)	Pos./Tested	Prev.% (95% CI)
Farm A	3/70	4.3 (0.9–12)	2/70	2.9 (0.3–9.9)
Farm B	6/32	18.8 (7.2–36.4)	5/32	15.6 (5.3–32.8)
Farm C	0/35	0 (0–10)	0/35	0 (0–10)
Farm D	6/76	7.9 (3–16.4)	6/76	7.9 (3–16.4)
Farm E	0/103	0 (0–3.5)	0/103	0 (0–3.5)
Farm F	7/90	7.8 (3.2–15.4)	5/90	5.6 (1.8–12.5)
Farm G	9/194	4.6 (2.1–8.6)	8/194	4.1 (1.8–8)
Farm H	0/23	0 (0–14.8)	0/23	0 (0–14.8)
Farm I	0/75	0 (0–4.8)	0/75	0 (0–4.8)
Farm J	1/46	2.2 (0.1–11.5)	1/46	2.2 (0.1–11.5)
Farm K	0/84	0 (0–4.3)	0/84	0 (0–4.3)
Total	**32/828**	**3.9 (2.7–5.4)**	**27/828**	**3.3 (2.2–4.7)**

RBPT-based prevalence differ significantly among sampled farms, $\chi^2 = 39.680$, $p < 0.001$. iELISA-based prevalence differs significantly among sampled farms, $\chi^2 = 33.498$, $p < 0.001$.

Table 3. Univariable in cattle and buffaloes at animal level.

Variable	Category	Pos./Tested	Prev.% (95% CI)	Odds Ratio	95% CI	*p*-Value *
Species	Cattle	9/419	2.2 (1–4)	Ref	-	0.012
	Buffaloes	23/409	5.6 (3.6–8.3)	2.71	1.24–5.94	
Breed	Local	32/790	4.1 (2.8–5.7)	-	-	-
	Cross	0/38	0 (0–9.3)	-	-	
Sex	Female	32/779	4.1 (2.8–5.7)	-	-	-
	Male	0/49	0 (0–7.3)	-	-	
Age groups	<2 Years	2/172	1.2 (0.1–4.1)	Ref	-	0.056
	≥2 Years	30/656	4.6 (3.1–6.5)	4.07	0.96–17.22	
Ticks infestation	No	31/766	4.1 (2.8–5.7)	2.57	0.35–19.17	0.356
	Yes	1/62	1.6 (0–8.7)	Ref	-	
RFM	No	29/781	3.7 (2.5–5.3)	Ref	-	0.363
	Yes	3/47	6.4 (1.3–17.5)	1.77	0.52–6.03	
History of abortion	No	30/771	3.9 (2.6–5.5)	Ref	-	0.885
	Yes	2/57	3.5 (0.4–12.1)	1.11	0.26–4.78	

RFM—Retention of fetal membranes; Ref—Reference value; * p value ≤ 0.05 considered as significant.

Table 4. Multivariable analysis at animal level for cattle and buffaloes.

Variable	Exposure Variable	Comparison	Odds Ratio	95% CI	*p*-Value *
Species	Buffaloes	Cattle	2.63	1.20–5.77	0.016
Age group	≥2 years	<2 years	3.89	0.92–16.47	0.065

* p value ≤ 0.05 considered as significant; (Model fitness: Nagelkerke R^2 (NR^2) = 0.051, Hosmer and Lemeshow Test (HLT) = 1.028, $p = 0.598$).

Samples from Farm C, Farm E, Farm H, Farm I, and Farm K did not show any positive by serology hence were not subjected for DNA extraction and molecular detection of *Brucella*-DNA. Out of total, 27 seropositive samples, 9 samples (6 buffaloes and 3 cattle) did amplify *Brucella*-DNA by conventional and subsequently *B. abortus*-DNA by real-time PCR.

In total, 828 serum samples were tested through RBPT and iELISA. Out of these, 32 samples were found positive in RBPT and 27 in iELISA (Table 2). Out of the 32 RBPT positive samples, 26 were

iELISA positive also. (Table 5). The agreement between RBPT and iELISA results was found almost perfect (k = 0.877) (Table 6).

Table 5. Comparison of results of RBPT and iELISA tests used to detect anti-*Brucella* antibodies in cattle and buffaloes.

RBPT		iELISA		Total
		Negative	Positive	
Negative	Count	795	6	801
	Expected Count	770	31	801
Positive	Count	1	26	27
	Expected Count	26	1	27
Total	Count	796	32	828
	Expected Count	796	32	828

Table 6. Agreement between RBPT and iELISA tests used for sero-diagnosis of brucellosis in cattle and buffaloes (n = 828).

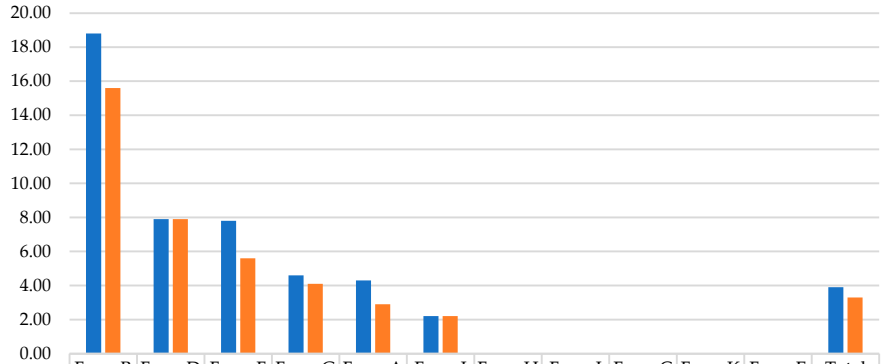
Comparison	Observed Agreement	SE	Kappa Value	95% CI of Kappa	*p*-Value *
RBPT vs. iELISA	99.15%	0.046	0.877	0.787, 0.967	<0.01

SE—Standard error; * p value < 0.05 considered as significant

4. Discussion

Serology remains an important tool in brucellosis diagnosis and RBPT and iELISA were used for screening of bovine sera in this study. RBPT has been widely accepted as a test with higher sensitivity and lower specificity as it can potentially cross-react with antibodies to other non-*Brucella* antigens [29]. Meanwhile, the iELISA is considered to be sensitive and could be used as a single diagnostic criterion at standardized labs [30]. However, RBPT remains an adequate screening test based upon the disease epidemiology, purpose of the diagnostic criteria and availability of the resources [31,32]. Therefore, we tested our sera by both tests and determined the possible agreement between these two tests (Tables 5 and 6). These serological tests do not differentiate between the *Brucella* species as *B. abortus*, *B. melitensis* and *B. suis* share common antigenic LPS. DNA-based tests, e.g., PCR, are able to differentiate at species level with high specificity. Clinical samples (e.g., serum and milk) contain lower amounts of bacterial DNA hence the sensitivity of PCR becomes really low. As the amount of bacterial DNA may depend upon the stage of the infection e.g., in chronic cases it is very unlikely to detect *Brucella*-DNA in serum samples. Real-time PCR provides a robust diagnostic solution with higher sensitivity, but also requires higher costs for the performance of this test. Isolation of Brucellae remains the gold standard for brucellosis diagnosis, but is less efficient, laborious and requires advanced laboratory conditions, e.g., level 3 biosecurity laboratories. A SYBR® Green-based assay was thus used for confirmation and differentiation of the etiology at species level.

In comparison to RBPT, iELISA and other diagnostic tests, similar results, as obtained in our study, were found previously in Pakistan [33]. However, statistically significant (p < 0.05) lower seroprevalence rates were detected by RBPT compared to iELISA [21]. This variability might be due to the difference in number and infection status of sampled animals, consumables used, laboratory conditions and personal expertise. Among the sampled farms, RBPT- and iELISA-based seroprevalence differed statistically significant (p < 0.05) ranging 0–18.8% and 0–15.6%, respectively. Although iELISA-based seroprevalence was found to be slightly lower than that of RBPT, the seroprevalence trend was the same at the farms for both RBPT and iELISA (Table 2). Highest seroprevalence 18.8% and 15.6% was found at Farm B followed by Farm D, Farm F, Farm G, Farm A, and Farm J by RBPT and iELISA, respectively (Figure 2).



	Farm B	Farm D	Farm F	Farm G	Farm A	Farm J	Farm H	Farm I	Farm C	Farm K	Farm E	Total
RBPT Overall %	18.80	7.90	7.80	4.60	4.30	2.20	0.00	0.00	0.00	0.00	0.00	3.90
iELISA Overall %	15.60	7.90	5.60	4.10	2.90	2.20	0.00	0.00	0.00	0.00	0.00	3.30

Figure 2. Farm-wise seroprevalence of brucellosis. RBPT—Rose Bengal Plate Agglutination Test; iELISA—Indirect Enzyme-Linked Immunosorbent Assay.

The seroprevalence pattern for buffaloes and cattle based on the location of the farms varied statistically significant ($p < 0.05$) (Table 1). Farm B, Farm F, Farm D, and Farm A had seropositive buffaloes whereas only Farm G and Farm J had seropositive cattle. Herd size, farm management practices, and contact with other domestic animals have been associated with the infection occurrence at different farms/herds [3,29,34,35]. However, the results are contradicted [36–38] and remain undetermined elsewhere in the country [39,40].

A variability in seroprevalence has been observed at institutional-owned, private-owned, general livestock population and rural animal holdings in Pakistan previously, based on these tests [21,35,36,41,42]. Brucellosis is an established professional health hazard in Pakistan [11,43–45]. Both *B. abortus* and *B. melitensis* have been identified [2–5,46–48]. Despite a great influx of brucellosis reporting in the recent past, livestock holders seem to be unaware of the infection [35]. Brucellosis is frequently reported at intensive dairy farms as compared to small animal holders in the country [29]. At the farms level, institutional-owned livestock farms tend to be less susceptible to the infection, maybe because of better screening, culling, hygiene and veterinary health facilitation programs than private livestock farms and a statistically significant difference ($p < 0.05$) has been reported [21,34,39,41], however disagreement does exist [42]. One of the major causes of brucellosis outbreaks especially at private-owned farms is the breach in biosecurity, i.e., the introduction of carrier animals (i.e., most often subclinical infected animals) into the existing herd without prior screening [4,49]. The infection remains unsuspected until abortion storm occurs and/or animals are screened for brucellosis. Brucellae do respond well to most of the commercially available antimicrobial agents, routine disinfectants and sterilization techniques although hints of resistance are reported [50,51]. They are killed by UV/sunlight exposure, 70% ethyl alcohol and by autoclavation [52,53]. Animals often conceive subsequently but remain carriers for their life. Veterinarians, municipal workers, butchers, technicians and householders acquire the infection unintentionally during unprotected handling of the infected animals [12,54].

More seropositive samples were found among the buffaloes i.e., 5.6% (23/409) and 4.7% (19/407) by iELISA than among cattle 2.2% (9/419) and 1.9% (8/419) by RBPT and iELISA, respectively, and that was statistically significant (Table 2). This difference is further clarified by multivariate analysis where buffaloes depict higher risk odds ratios than cattle for the infection (Table 4). Similar statistically significant results have been reported previously [21,36,55] however, contradictive results by Seed et al. [3] and without statistical determination are also reported [21,40]. To the best of our understanding, the real reason for biological affinity of buffaloes towards brucellosis remains unclear.

Although our study could not find statistically significant association for breed of the animals with brucellosis, the crossbred and exotic cattle have been previously reported to be more prone to the infection as compared to the local/indigenous breeds [49,56–59]. Specifically, within the cattle,

breeds, i.e., Sahiwal, Cholistani, and crossbred, univariate analysis did not show statistical significance ($p > 0.05$) with the infection (Table 3). This might be due to the difference in geography or sampling bias because of the presence of a higher number of local/indigenous cattle population at these farms. Nevertheless, *Nramp1* gene is associated with brucellosis resistance [60–62].

Our study found only females positive for brucellosis and could not determine a statistical association, although sex of the animals was not associated statistically significant ($p > 0.05$) in previous reports [21,33,41] although associated by Ali et al. [36]. This may be due the fact that relatively fewer bulls are kept at dairy purpose farms because of increasing local artificial insemination facilities and interest of the farm owners in female animals for production [21]. However, controversial arguments do exist [63].

More animals were tested positive in age group ≥ 2 years but were found statistically non-significant ($p > 0.05$) to the infection as supported by the previous findings [3,33,36,41]. Similar results are reported in cattle but a statistically significant association was found in buffaloes [55]. A similar trend was observed with the increase in age, but statistical significance was not determined [40]. However, mature animals remain at higher risks [36]. Young animals contract the infection when fed on contaminated colostrum or milk from infected dames. Although our study analyzed the relation of presence of ticks with brucellosis, a statistically non-significant relation was found. Similarly, the multivariate analysis did not show any statistically significant association ($p > 0.05$) (Table 4). External parasites and ticks have not been related to brucellosis epidemiology so far [52].

RFM and history of abortion did not show statistically significant association ($p > 0.05$) to the infection in our study, maybe because of the better health and husbandry services at these farms. However, this observation has been contradicted by previous reports that have found a significant association [3,33,36,38,55].

5. Conclusions

Brucellosis remains a persistent infection in bovines in Pakistan. Husbandry practices might play a role determining the occurrence of the infection at a specific farm/location. Buffaloes seem to be at higher risk when compared to cattle. Although, specific breed, sex of the animals, age and history of reproductive disorders could not be associated in the study, based on previous literature, these factors should not be ignored while screening for brucellosis. *B. abortus* was detected to be the cause of infection. Small ruminants as well as non-preferred hosts (dogs, equines, etc.) in close contact are needed to be tested to determine the presence/transmission role of these animals to the infection. A standardization of the diagnostic system, e.g., ELISA and PCR, is recommended. Routine diagnostic screening, culling, biosafety, biosecurity, and quarantine measures are needed to continue especially when introducing new animals to the existing herd. The milk chain is needed to be traceable at these farms to avoid unintentional mixing of contaminated/antimicrobial-treated milk into the main supply chain to avoid human transmission. The pasteurization of milk would be highly recommended. Proper disinfection and sterilization of the area and personal protection is needed in case of abortion outbreaks at farms. Isolation and identification of the etiological agents at molecular level is recommended when required facilities are available. Based on the results in this study, RBPT can be used sufficiently for the purpose of screening for brucellosis in farm animals under local conditions. This study is the first in which *Brucella* was identified to the species level at organized institutional livestock farms in Pakistan.

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3.2 Publication 2

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Article

Serological and Molecular Investigation of *Brucella* Species in Dogs in Pakistan

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Abstract: Brucellosis is an important bacterial zoonosis caused by *B. abortus* and *B. melitensis* in Pakistan. The status of canine brucellosis caused by *B. canis* remains obscure. In total, 181 serum samples were collected from stray and working dogs in two different prefectures viz. Faisalabad (n = 87) and Bahawalpur (n = 94). Presence of antibodies against *B. canis* and *B. abortus*/*B. melitensis* was determined using the slow agglutination test (SAT) and ELISA, respectively. Real-time PCR was performed to detect and differentiate *Brucella* DNA at the species level. In Faisalabad, the serological prevalence was found to be 9.2% (8/87) and 10.3% (9/87) by SAT and ELISA, respectively. Only one of the ELISA positive samples (1.15%) yielded amplification for *B. abortus* DNA. In Bahawalpur, 63.8% (60/94) samples were found positive by SAT; however, none of the samples was positive by ELISA or by real-time PCR. Location, age (≥1 year) and body condition (weak) were found to be associated with *B. canis* infection, whereas presence of wounds was found to be associated with *B. abortus* infection only. These findings point towards a risk of transmission from dog to livestock and humans and vice versa. The study expects to draw the attention of concerned authorities towards infection prevention and animal welfare. This study warrants further epidemiological investigation on brucellosis in pet dogs and their owners. To the best of our knowledge, this is first ever report on *B. canis* and *B. abortus* in dogs in Pakistan.

Keywords: Dogs; *Brucella abortus*; *Brucella canis*; zoonosis; Pakistan

1. Introduction

Brucellosis is a serious bacterial zoonosis caused by *Brucella (B.)* species. It affects a wide range of wild and domestic animals worldwide. Of the 12 accepted nomo-species of *Brucella*, at least *B. abortus* (primary host: *Bovidae*), *B. melitensis* (small ruminants), *B. suis* (pigs) and to some extent *B. canis* (*Canidae*) are known human pathogens [1,2]. In domestic animals, abortion, retained placenta, orchitis and rarely arthritis are the cardinal symptoms that result in serious economic losses to the livestock industry [3]. Brucellosis poses a significant emerging threat to public health owing to the lack of vaccines, limited treatment options and significant number of relapses [4]. Microbiological diagnosis

of brucellosis remains challenging, as isolation of the etiologic agent is hazardous and restricted to specialized laboratories, e.g., biosafety level 3. Thus, diagnosis relies mainly on serology. Treatment in farm animals is forbidden in many countries and vaccines are not always protective and safe for human health [5]. Eradication programs strictly follow test and slaughter/culling policy. Dogs are susceptible to *B. melitensis* and *B. suis* and could remain asymptomatic carriers for *B. abortus* [6–8]. Canine brucellosis caused by *B. canis* is manifested by late abortion and retention of fetal membranes in female dogs and orchitis, epididymitis and testicular atrophy in male dogs. Infected animals shed bacteria in body secretions viz. vaginal fluid, semen, saliva, nasal and ocular secretions, feces and milk and can transmit the infection directly through contact or indirectly via contamination of the environment [1,9,10]. Canine brucellosis is communicable to humans and other animal species and infections have been reported in different parts of the world [11]. Largely, brucellosis in dogs is considered as an under-estimated hazard to human health and animal welfare [12].

In Pakistan, brucellosis is considered endemic in ruminants and *B. abortus* and *B. melitensis* have been isolated from bovines and small ruminants, respectively [13–15]. Human brucellosis is well reported and has been described as a professional health hazard [16–18]. However, knowledge on the status of infection and possible epidemiological role of non-ruminant domestic animals (equine, canine, and feline) and wildlife in brucellosis remains scarce in the country [19–22].

A tremendous increase in popularity of keeping dogs as pets has been seen in Pakistani society over the last two decades [23]. Existence of a strong human–animal (dog) bond may pose a serious risk of transmission of brucellosis, especially among dog keepers. Serological tests that detect smooth lipopolysaccharide (LPS) of *Brucella* (*B. aboruts, B. melitensis* and *B. suis*) do not detect rough LPS (*B. canis* and *B. ovis*) [24]. Previous studies are limited to serological detection of livestock brucellosis in dogs [19]. Thus, we designed the current study to investigate the prevalence of antibodies against the smooth LPS antigen of *B. abortus* and *B. melitensis*, the rough LPS antigen of *B. canis* in dog sera, and the possible detection/differentiation of *Brucella* DNA at the species level to precisely identify the etiology and to determine related risk factors for the corresponding infection.

2. Results

A total of 37.6% (68/181) samples were found to be seropositive for canine brucellosis (*B. canis*) by slow agglutination test (SAT) and 4.9% (9/181) for livestock brucellosis (*B. abortus* and *B. melitensis*) by ELISA. The seroprevalence of *B. canis* was significantly higher in the dogs from Bahawalpur (63.8%, confidence interval (CI) 53.3–73.5) as compared to those from Faisalabad (9.2%, CI 4.1–17.3), Chi-square (χ^2) = 56.55, $p < 0.001$. Using PCR, only one sample originating from the Faisalabad region was detected as positive for *B. abortus*.

Of the 94 serum samples collected from Bahawalpur, 60 (63.8%) were positive by SAT (Table 1) which subsequently tested negative by ELISA and real-time PCR. Among 87 serum samples originating from Faisalabad, eight (9.2%) were found positive by SAT and nine (10.3%) by ELISA. One ELISA positive sample amplified *B. abortus* DNA by real-time PCR. As no amplification was found for *B. melitensis*, we assumed livestock brucellosis was caused by *B. abortus* in these dogs.

Location, age and body condition were the variables that showed significant ($p < 0.05$) association with *B. canis* in dogs. In the univariable analysis, dogs from Bahawalpur (odds ratio (OR) 17.43, CI 7.52–40.37), between 1–2 years of age (OR 3.96, CI 1.73–9.06), above two years of age (OR 3.09, CI 1.29–7.39) and with weak body condition (OR 2.73, CI 1.45–5.16) were found likely to test positive for *B. canis* (Table 1). In the multivariate analysis, location and age factors were found to be associated with *B. canis* prevalence. The model showed that dogs from Bahawalpur (OR 16.41, CI 6.99–38.53), between 1–2 years of age (OR 3.12, CI 1.19–8.15) and >2 years of age (OR 2.94, CI 1.06–8.17) were more likely to test positive for *B. canis* (Table 2). Sex, contact with other animals, presence of wounds, presence of ticks and external parasites, fever, and eye condition were excluded from the multivariate model at different steps as these variables did not show statistical association ($p > 0.05$) with infection (Table 1).

Table 1. Univariate analysis of risk factors for canine brucellosis (*B. canis*).

Variable	Category	Positive/Tested	Prevalence, % (95% CI)	OR (95% CI)	*p* Value *
Location	Faisalabad	8/87	9.2 (4.1–17.3)	Ref	<0.001
	Bahawalpur	60/94	63.8 (53.3–73.5)	17.43 (7.52–40.37)	
Contact with animals	No	28/61	45.9 (33.1–59.2)	1.70 (0.90–3.19)	0.1
	Yes	40/120	33.3 (25–42.5)	Ref	
Sex	Male	44/123	35.8 (27.3–44.9)	Ref	0.468
	Female	24/58	41.4 (28.6–55.4)	1.27 (0.67–2.40)	
Age groups	<1 year	10/53	18.9 (9.4–32)	Ref	0.004
	1–2 years	35/73	47.9 (36.1–60)	3.96 (1.73–9.06)	
	>2 years	23/55	41.8 (28.7–55.9)	3.09 (1.29–7.39)	
Body condition	Weak	33/62	53.2 (40.1–66)	2.73 (1.45–5.16)	0.002
	Normal	35/119	29.4 (21.4–38.5)	Ref	
Wounds	No	40/120	33.3 (25–42.5)	Ref	0.1
	Yes	28/61	45.9 (33.1–59.2)	1.70 (0.90–3.19)	
Tick infestation	No	41/96	42.7 (32.7–53.2)	1.60 (0.87–2.95)	0.13
	Yes	27/85	31.8 (22.1–42.8)	Ref	
Ecto-parasites	No	47/116	40.5 (31.5–50)	1.43 (0.75–2.70)	0.275
	Yes	21/65	32.3 (21.2–45.1)	Ref	
Fever	No	57/152	37.5 (29.8–45.7)	Ref	0.965
	Yes	11/29	37.9 (20.7–57.7)	1.02 (0.45–2.31)	
Eye condition	Normal	52/132	39.4 (31–48.3)	3.25 (0.37–28.61)	0.515
	Red	15/43	34.9 (21–50.9)	2.68 (0.29–25.08)	
	Ulcer	1/6	16.7 (0.4–64.1)	Ref	
Total		68/181	37.6 (30.5–45.1)		

* p value < 0.05 considered as significant.

Table 2. Multivariate analysis of risk factors for canine brucellosis (*B. canis*).

Variable	Exposure Variable	Comparison	OR	95% CI	*p* Value
Location	Bahawalpur	Faisalabad	16.41	6.99–38.53	<0.001
Age group	1–2 years	<1 year	3.12	1.19–8.15	0.049
	>2 years	<1 year	2.94	1.06–8.17	

Model Fit: Nagelkerke R^2 = 0.435, Hosmer and Lemeshow Test (χ = 4.004, p = 0.405).

The presence of wounds (OR 4.26, CI 1.03–17.65) showed significant association ($p < 0.05$) with *B. abortus* prevalence in the univariate analysis. Location and contact with other animals could not be analyzed as no positive samples were found in Bahawalpur. All other variables did not show significant association ($p > 0.05$) to the infection, and hence multivariate analysis could not be performed (Table 3).

Table 3. Univariate analysis of risk factors for livestock brucellosis (*B. abortus*).

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	*p* Value *
Location	Faisalabad	9/87	10.3 (4.8–18.7)	-	-
	Bahawalpur	0/94	0 (0–3.8)	-	
Contact with animals	No	0/61	0 (0–5.9)	-	-
	Yes	9/120	7.5 (3.5–13.8)	-	

Table 3. *Cont.*

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	*p* Value *
Sex	Male	8/123	6.5 (2.8–12.4)	3.97 (0.48–32.48)	0.199
	Female	1/58	1.7 (0–9.2)	Ref	
Age groups	<1 year	1/53	1.9 (0–10.1)	Ref	0.278
	1–2 years	6/73	8.2 (3.1–17)	4.66 (0.54–39.89)	
	>2 years	2/55	3.6 (0.4–12.5)	1.96 (0.17–22.31)	
Body condition	Weak	1/62	1.6 (0–8.7)	Ref	0.167
	Normal	8/119	6.7 (2.9–12.8)	4.40 (0.54–35.98)	
Wounds	No	3/120	2.5 (0.5–7.1)	Ref	0.046
	Yes	6/61	9.8 (3.7–20.2)	4.26 (1.03–17.65)	
Tick infestation	No	6/96	6.3 (2.3–13.1)	1.82 (0.44–7.52)	0.407
	Yes	3/85	3.5 (0.7–10)	Ref	
Ecto-parasites	No	6/116	5.2 (1.9–10.9)	1.13 (0.27–4.67)	0.869
	Yes	3/65	4.6 (1–12.9)	Ref	
Fever	No	7/152	4.6 (1.9–9.3)	Ref	0.605
	Yes	2/29	6.9 (0.8–22.8)	1.53 (0.30–7.79)	
Eye condition	Normal	7/132	5.3 (2.2–10.6)	1.15 (0.23–5.75)	0.986
	Red	2/43	4.7 (0.6–15.8)	Ref	
	Ulcer	0/6	0 (0–45.9)	-	
Total		9/181	5 (2.3–9.2)		

* p value < 0.05 considered as significant.

3. Discussion

Brucellosis remains a persistent zoonotic infection mainly caused by *B. abortus* and *B. melitensis* in ruminants in Pakistan and neighboring countries [25–33]. Canine brucellosis (*B. canis*) has also been reported in surrounding countries [34–36]. Faisalabad is the third largest city and is one of the leading districts in terms of daily milk production in the country. It bears a total of 2.7 million livestock heads; a mostly bovine population [37]. Brucellosis has been reported from Faisalabad in bovines, equines, camels and humans [38–40]. Bahawalpur is a relatively smaller city with a livestock population of 2.4 million heads, mostly small ruminants raised as nomadic/pastoral herds [37]. Only a few reports exist for brucellosis in bovines from Bahawalpur [38,40].

Serology is a main stay of brucellosis diagnosis and nonspecific reactions are not uncommon [41]. ELISA is a reliable diagnostic solution for livestock brucellosis (*B. abortus/B. melitensis/B. suis*) [42]. *B. canis* is of the rough LPS type and standard diagnostics for livestock brucellosis cannot be used. However, lower sensitivity of the antigen preparations of *B. canis* hampers diagnosis [43]. Real-time PCR assays may detect cases with negative serology [44]. No specific real-time assay for *B. canis* has been established yet. For livestock brucellosis, simultaneous serology and real-time PCR assays were applied to detect and differentiate *B. abortus* and/or *B. melitensis*. We used an in-house prepared *B. canis* antigen in SAT for the diagnosis of canine brucellosis. Besides SAT, a genus specific real-time assay was used.

The size of the dog population is unknown in Pakistan [45,46]. Dogs are mainly kept for watch purposes and to a lesser extent for companionship and fighting competitions [23]. Dogs kept at dairy farms/households in rural areas are used to guard the animals inside and outside of the dairy farms and during grazing. It is challenging to differentiate these rural household dogs from stray dogs as

they often roam freely in the nearby countryside. Both of these types of dogs often have access to the rejected flesh from slaughterhouses, butcheries or municipal dumps, dead animal carcasses and remains of livestock, e.g. placentas or aborted fetal material, and also to kitchen leftovers [47–49].

The prevalence of antibodies to *B. canis* varied statistically significant ($p < 0.05$) among dogs from Faisalabad and Bahawalpur. Dogs from Bahawalpur were found to be more likely (OR 16.41, CI 6.99–38.53) to have canine brucellosis than those from Faisalabad (Tables 1 and 2). An alarmingly high number of SAT positive dogs 63.8% (60/94) were found negative by PCR. This may be attributed to the persistence of infection as well as by intensive breeding with few preferred but *B. canis* infected males and vice versa [9,10]. The relatively higher number of *B. abortus* cases in Faisalabad indicated that these dogs had regular contact with *Brucella* antigen, and chronic persistence of infection in some of these stray dogs may be present too. Recently, DNA from *B. abortus* was detected in soil in Faisalabad where animals and humans lived in proximity [50]. The detection of *B. abortus* DNA in a seropositive stray dog confirms the actual presence of infection in the dog population of Faisalabad. No such proof existed for Bahawalpur. It is known that *Brucellae* from livestock are transmissible to animals living in close contact. The role of farm dogs as a host for bovine brucellosis and a source of re-infection at the farm level has already been confirmed [51–54]. Thus, counter measures must also include dogs having access to farms. This is also true for *B. canis* as infected dogs shed *Brucellae* in milk, urine, feces, nasal and ocular secretions, as well as in their saliva, and a risk for transmission to humans and other animals can also be assumed [55–58].

For *B. canis*, age was found to be associated ($p < 0.05$) with a higher risk of dogs older than one year of age (Table 1). The highest risk was found in 1–2 year old dogs. A similar pattern was observed in European and Iranian dogs [34,59]. Age might reflect repeated contact with *B. canis* excreting conspecifics. For *B. abortus*, such an association was found in Africa [60], but could not be confirmed in our study.

Dogs with weak body condition bared a 2.73 (1.45–5.16: 95% CI) times higher risk than dogs with normal body condition for canine brucellosis seropositivity. This may be attributed to weaker immune systems and failure to compete with healthier dogs. A non-significant association was found for *B. abortus* infection (Tables 1 and 3).

The presence of wounds on the body indicated a 4.26 (1.03–17.65; 95% CI) times higher risk for being seropositive for livestock brucellosis (*B. abortus*) (Table 3). The meaning of this finding is not clear but may be connected to food acquisition. Based on our observations, these dogs feed on leftovers (placenta etc.) of bovines, fight with each other, get injured by physical barriers while entering feeding places or simply get hurt by people protecting farms or places where cadavers or discards are stored. Further studies can help to explain this finding.

A statistically non-significant ($p > 0.05$) association was found with the sex of the animals with seropositivity for both *B. canis* and *B. abortus*. A similar pattern was found in India but has remained unestablished in dogs from Jordan and Europe [59,61,62]. Similarly, a statistically non-significant association ($p > 0.05$) was found for tick and ecto-parasite infestation, fever and eye condition.

Although the number of dogs investigated is low, this study draws attention to the fact that brucellosis in stray dogs can be present. The low number of samples is due to the semi-wild lifestyle of the dog population, as blood sampling is simply often impossible and can be hazardous for the operator.

4. Materials and Methods

A total of 181 serum samples were collected from stray and working dogs based on convenient sampling: 87 serum samples were collected from Faisalabad and 94 from Bahawalpur districts in Punjab, Pakistan between December 2015–2016. Blood samples were collected under sterile conditions and serum was separated and kept at −20 °C until further analysis. The serum samples were sent to the Office International des Epizooties and National Reference Laboratory for Brucellosis at the Friedrich-Loffler Institute (FLI), Jena, Germany for serological and molecular diagnosis. All sera were tested by SAT using rough *B. canis* LPS antigen (FLI, Jena, Germany) as described by [63].

A titer >1:50 was considered as positive. The ID Screen® Brucellosis serum indirect multi-species enzyme linked immunosorbent assay (ELISA) for the detection of anti-smooth LPS antibodies of *B. abortus* and *B. melitensis* was performed following the manufacturer's recommendations (ID Vet, Garbels, France). Both districts were chosen as representative for different epidemiological settings of livestock brucellosis. Based on previous literature, we supposed that *B. melitensis* is prevalent in these districts although no isolates were available at the time of sample collection [64].

The sera were subjected to DNA extraction using the QIAamp DNA Mini Qiacube kit (Qiagen, Hilden, Germany) along with *E. coli* biomass as a negative control in each run. By using real-time PCR, molecular detection and differentiation of *Brucella* DNA (*B. abortus* and *B. melitensis*) was made [65]. For each run, positive controls of *B. abortus* (ATCC 23448) and *B. melitensis* (ATCC 23456) along with a no template control (NTC) of nuclease free water were included (see Table S1). PCR conditions were as follows: decontamination at 50 °C for 2 min, 1 cycle; initial denaturation at 95 °C for 10 min, 1 cycle; denaturation at 95 °C for 25 secs and 57 °C for 1 min for annealing and elongation of the primers, both for 50 cycles each. Samples showing cycle threshold (Ct) ≤38 were considered positive as based on in-house validation using camel sera [66].

For statistical analysis, the seroprevalence along with 95% exact binomial confidence interval (CI) was calculated. The Chi-square test ($\chi 2$) was used to determine significant differences. Furthermore, the data regarding different variables were subjected to univariable analysis to determine the association between the independent/explanatory variables obtained from the questionnaire and brucellosis seropositivity as the dependent/outcome variable. Odds ratio (OR), along with the respective CI, was calculated for different variables. All variables with the p value < 0.20 were included in the initial binary logistic regression model, and a backward stepwise approach was used to exclude the variable with the highest p value until all the confounders were removed [67]. The values of the Nagelkerke R^2 (NR^2) and Hosmer and Lemeshow Test (HL) were used to judge the fitness of the final model. Analysis was done using the SPSS software (IBM SPSS Statistics for Windows, Version 20.0).

5. Conclusions

Brucellosis in dogs is caused by *B. abortus* and *B. canis* in Faisalabad and Bahawalpur, Pakistan. *B. abortus* was confirmed by real-time PCR in dogs from Faisalabad but not in dogs from Bahawalpur. Geographical location seemed to play a role in the epidemiology of *B. canis* infection. Being older than one year of age and a weaker body condition were associated with *B. canis* but not with *B. abortus*. The presence of wounds on the body was associated with *B. abortus* infection only. Other factors such as sex, contact with other animals, ecto-parasite infestation, fever and eye condition were not associated with *B. canis* or *B. abortus* infection. Further studies may help in understanding the epidemiology of the infection. It becomes apparent that pet dogs and their owners have to be investigated to estimate the risk of human transmission. Humans in close contact with infected dogs should be tested for both *B. canis* and *B. abortus*/*B. melitensis*. Isolation and identification of *Brucellae* from clinically ill dogs and humans is important for molecular epidemiology. Up-to-date laboratory facilities and training is required for this purpose. Comprehensive studies may include all animals living near infected dogs and more than one diagnostic test should be used. Counter measures must include raising public awareness for canine brucellosis and routes of transmission to other animals and humans. Stray, wild and semi-wild canines must be included in brucellosis surveillance and eradication program. Although the study did not include clinically ill dogs, they should be included in future studies. Clinically healthy dogs may carry the infection sub-clinically, hence prior screening is necessary. To the best of our knowledge, this is the first study to test and report the presence of *B. canis* and *B. abortus* in dogs from Pakistan.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/8/4/294/s1, Table S1: Primer and probes sequence for real-time PCR.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical Statement: Blood and serum samples were collected as per ethical and animal welfare guidelines defined by the "Ethical review committee" of the College of Veterinary and Animal Sciences, University of Veterinary and Animal Sciences, Lahore, sub-campus Jhang, Pakistan (letter no. CVAS/849).

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3.3 Publication 3

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Epidemiology and associated risk factors for brucellosis in small ruminants kept at institutional livestock farms in Punjab, Pakistan.

Frontiers in Veterinary Science (under review).

Epidemiology and associated risk factors for brucellosis in small ruminants kept at institutional livestock farms in Punjab, Pakistan

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Abstract

Brucellosis is considered as an endemic infection in ruminants in Pakistan. Both *Brucella abortus* and *B. melitensis* infections have been reported in domestic animals and humans in the country. This study aimed to identify the burden of anti-*Brucella* antibodies in small ruminants as well as potential risk factors associated with its occurrence at nine institutional livestock farms. A total of 1000 sera (500 sheep and 500 goats) were collected. Samples were screened by indirect-ELISA for anti-smooth-*Brucella* antibodies. Overall, 5.1% (51/1000) of the sera were found seropositive for the antibodies with 5% (25/500) prevalence in goats and 5.2% (26/500) in sheep. *Brucella*-DNA was not detected by real-time PCR in any of the tested sera. No significant association was observed between seropositivity and sex or species of the animals. Binary logistic regression model indicated that small ruminants; kept at farm 2 (OR 34.05), > 4 years of age (OR 2.88), with history of reproductive disorders (OR 2.69), and with BCS of less than or equal to 3 (OR 12.37) were more likely to test positive for the brucellosis at these farms. We suggest that farm biosecurity and brucellosis-screening programs should be improved at these farms.

Keywords: sheep, goats, brucellosis, risk factors, Pakistan

Introduction

Brucellosis is a bacterial zoonosis with worldwide distribution, which is caused by bacteria of the genus *Brucella*. This genus is classified into; *B. melitensis, B. abortus, B. suis, B. canis, B. ovis* and *B. neotome* (classical *Brucella* species), *B. ceti* and *B. pinipedialis* from marine mammals, *B. microti* from voles, *B. inopinata* from human females, *B. papionis* from baboons and recently *B. vulpis* from red foxes (Foster et al., 2007; Garrity et al., 2004; Scholz et al., 2008; Scholz et al., 2010; Scholz et al., 2016; Whatmore et al., 2014). Based upon host preference; *B. abortus* predominantly infects bovines, *B. melitensis* small ruminants, *B. canis* dogs, *B. suis* pigs and *B. ovis* rams, however, infection in non-typical hosts is transmissible (Jamil et al., 2019; Saeed et al., 2019; Saleem et al., 2019). Greater prevalence is observed in developing and tropical countries where it causes abortion and retention of fetal membranes (Corbel, 2006). The infection may stay undiagnosed, as it can remain asymptomatic and animals may conceive subsequently but remain carriers for their life. The infection is of economic importance, especially in developing countries (McDermott et al., 2013). Direct or indirect contact with diseased animals and consumption of contaminated raw milk and products are the main routes of transmission in animals and humans respectively (Gul and Khan, 2007). It is an established occupational health hazard (Ali et al., 2013; Asif et al., 2014; Mukhtar, 2010; Mukhtar and Kokab, 2008). Diagnosis remains a challenge and is based mostly on serology and milk ring test (MRT). Molecular biological detection of *Brucella*-DNA in clinical samples, e.g. by polymerase chain reaction (PCR), is coupled to identify the etiology precisely where necessary. Isolation of bacterium is a gold standard for the diagnosis of brucellosis but requires specific growth conditions. Moreover, fastidious nature of the organism makes primary isolation of *Brucella* difficult. Vaccination is recommended but has limited practice in developing countries such as in Pakistan (Nawaz et al., 2016). Treatment for brucellosis in farm animals is also not very popular in the country hence, test and slaughter/culling policy remains a sole solution for eradication of the infection in farm animals.

Pakistan is an agriculture-based country in south-Asia, where livestock plays a vital role in the national economy. The total livestock population in the country is 142.8 millions, where small ruminants (sheep and goat) share 80.27 million heads (Anonymous, 2006). In the past, brucellosis has been reported in both large and small ruminants in Punjab, Pakistan (Arshad et al., 2011; Gul et al., 2015; Gul et al., 2014; Iqbal et al., 2013; Jamil et al., 2020). This study was aimed to ascertain the current status of brucellosis in small ruminants at institutional livestock farms located in Punjab. Additionally, we determined the risk factors associated with the occurrence of the disease.

Materials and Methods

A total of 1,000 sera (500 each from sheep and goats) were collected from nine different institutional livestock farms maintained under the Livestock and Dairy Development Department (L&DD), Government of Punjab, Lahore, Pakistan (Figure 1) (Ullah et al., 2019). The sample size was calculated for an estimated disease prevalence of 50% at a 95% confidence interval, and 5% desired absolute precision (Table 1) (Thrusfield, 2007). A minimum of 384 samples from each species were required by this method. The sample size was further inflated to accommodate for the potential losses during the transportation. The final sample size was proportionally allocated to each farm according to the population of the animals at each farm. Available identification record was used at each farm, to randomly select animals by using a random number generator. Individual animals were restrained, and blood was collected in a 9 mL vacutainer tube without anticoagulant through the jugular vein. No animals were harmed during this process. The animals had no prior history of brucellosis vaccination.

Sera were screened by ID Screen® Brucellosis Serum Indirect Multi-species (IDVet, Grabels, France) indirect-ELISA for detection of anti-smooth-lipopolysaccharide (LPS) (*B. abortus, B. melitensis* and *B. suis*) antibodies at the National Reference Laboratory (NRL) for brucellosis, Friedrich-Loeffler-Institut (FLI), Jena, Germany as per manufacturer's recommendations. DNA was extracted from sera by using the High Pure Template Kit (Roche, Rotkreuz, Switzerland) and molecular detection was done by real-time PCR as described by Probert et al. (Probert et al., 2004). The DNA extraction was run along with *E. coli* controls. The real-time PCR was run along with *B. abortus* (ATCC 23448) and *B. melitensis* (ATCC 23456) as positive controls. For no template negative control (NTC), nuclease-free water was used.

Brucellosis prevalence at species level was calculated by dividing the number of positive animals (numerator) by the total number of animals sampled (denominator). Univariate and multivariate analysis were conducted to determine the association of the risk factors with the seroprevalence. Chi-square test (χ^2) of independence was used to compute the significance of association. The univariate analysis was conducted for farm related and animal level variables. Brucellosis was considered as an outcome or dependent variable while biological plausible variables e.g. farm location, species, sex, age/parity status, breed, history of reproductive disorders and body condition score (BCS) were considered as explanatory or independent variables. $P \leq 0.05$ was considered as a level of significance. A backward stepwise approach was used for the binary logistic regression analysis (Bursac et al., 2008). Nagelkerke R^2 (NR^2) and Hosmer and Lemeshow test (HLT) were used to assess the model-fitness. The statistical analysis was conducted using the IBM SPSS Statistics (IBM Corporation, Armonk,

New York, USA). The maps were generated by using ArcGIS version 10.5.1 (ESRI, Redlands, CA, USA).

Results

In total, 51 (5.1%, CI 3.8-6.7) samples were found positive for the antibodies against *Brucella*. The seroprevalence was almost identical in goats (5.2%) and sheep (5.0%), p=0.886 (Tables 1 and 2). Seropositive animals were detected at the five out of nine sampled farms, and the prevalence varied from 2.1% (Farm 9) to 48.7% (Farm 2), p<0.001. In goats, the highest seroprevalence was recorded in the small ruminants at Farm 5 (15.9%) and the lowest at the Farm 9 (2.9%), p=0.001. In sheep, the seropositivity ranged from 2.5% (Farm 4) to 48.7% (Farm 2), p<0.001 (Figure 1). None of the samples contained *Brucella* DNA as confirmed by negative real-time PCR results.

The univariable analysis indicated that sheep at Farm 2 were significantly (p<0.001) more likely to test positive for anti-*Brucella* antibodies (OR 25.7, CI 12.84-55.52). In females, the seropositivity (5.3%) and odds for testing positive (OR 1.43, 0.51-4.05) were higher as compared to males (3.7%), p=0.5. The small ruminants; above 4 years of age (7.9%, OR 2.94 CI 1.60-5.38), of multiparous status (6.7%, OR 2.59 CI 1.31-5.12), belonging to Buchi breed (48.7%, OR 26.7 CI 12.84-55.52), with history of reproductive disorders (13.6%, OR 3.19 CI 1.29-7.95) and having BCS ≤3 (19.8%, OR 11.74 CI 6.39-21.62) were found significantly ($p < 0.05$) more likely to test seropositive (Table 2).

The multivariable analysis indicated that small ruminants; kept at Farm 2 (OR 34.05 CI 13.47-86.10), above four years of age (OR 2.88 CI 1.39-5.94), with history of reproductive disorders (OR 2.69 CI 1.33-5.42) and BCS ≤3 (OR 12.37 CI 5.98-25.57) were significantly ($p < 0.01$) more likely to test positive for anti-*Brucella* antibodies. The values of Nagelkerke R^2 (0.407) and Hosmer and Lemeshow test (Ci-square value; $\chi 2 = 3.092$, $p = 0.543$) indicated that it was a reasonable model to predict seroprevalence of brucellosis at the sampled farms.

Discussion

Brucellosis remains an endemic infection in livestock in Pakistan (Farooq et al., 2011; Nawaz et al., 2016). Serology is a preferred choice for diagnosis of brucellosis. RBPT is a sensitive and cheaper test and is widely used for screening of brucellosis in developing countries like Pakistan. The major shortcoming of this test is that it can react non-specifically to antibodies against other Gram-negative bacteria and cannot differentiate between vaccinated and infected animals (Nielsen and Yu, 2010). ELISA, a sensitive test, is also unable to differentiate between vaccinated and infected animals but is useful for diagnostic screening on larger scale (Uzal et al., 1995). Molecular biological tests, e.g. PCR, focus on the presence of DNA in the sample and are potentially able to differentiate the vaccine and field strains of *Brucella* (Lopez-

Goñi et al., 2008). Real-time PCR can even detect and differentiate at lower amounts of DNA in a clinical sample when compared to conventional PCR. However, it requires the presence of bacterial DNA in the sample, which may not be present at every time during and after an infection and might be affected by laboratory procedures (Jamil et al., 2017). Hence, we used indirect-ELISA as a single screening test and real-time PCR for confirmation of the etiology.

Among variables, the odds for testing positive were significantly higher in the animals kept at Farm 2 (Table 2 and Table3). From two types of small ruminants targeted in our study, only sheep were present at this farm. Location and geography have been reported to be associated with *Brucella* infection in small ruminants (Gul et al., 2015; Iqbal et al., 2013; Naeem and Kamran, 2013), which could be related to the differences in sampling area and herd management system. Furthermore, small ruminants had a close contact with bovines at Farms (2, 5, 6, 7, and 8), where brucellosis was reported previously (Gul et al., 2014; Jamil et al., 2020; Nasir et al., 2004). Moreover, common grazing and watering points, use of brucellosis positive male for breeding and introduction of new animals without testing etc. could be other factors responsible for the brucellosis incidence at these locations (Cárdenas et al., 2019; Ullah et al., 2015).

We did not find any association of seroprevalence with species of small ruminants in our study, and an almost identical seroprevalence of brucellosis was detected at individual level in both species. Furthermore, 75% (3/4) farms having goats were positive, and 40% (2/5) farms only with sheep were found positive for brucellosis in our study. However, this difference in seroprevalence was not significant ($p>0.05$). Various studies reported higher seroprevalence in goats; without reporting the significance of association (Ali et al., 2017; Ali et al., 2013; Din et al., 2013; Ghani et al., 1995; Nasir et al., 2000; Qureshi and Masood, 1988), with non-significant association (Khan et al., 2017) and with significant association (Gul et al., 2014). In contrast, a higher seroprevalence in sheep than in goats has been reported elsewhere (Saleem et al., 2019; Naeem and Kamran, 2013). Most of these reports used RBPT test for the screening of brucellosis. A more healthy and diverse sampling is required to find out if either of species is more susceptible to brucellosis.

Although female animals showed higher seroprevalence rate of brucellosis in our study, sex of the animals was not found associated with seropositivity ($p>0.05$). These findings agree with previous studies (Ali et al., 2017; Ali et al., 2015; Arshad et al., 2011; Din et al., 2013; Ghani et al., 1995; Hussain et al., 2014; Khan et al., 2017). There are studies reporting the same or higher seroprevalence rate in males with or without statistical association (Gul et al., 2014; Iqbal et al., 2013). The reason for higher incidence rates in females might be that female animals are more studied, interest of owners due to production parameters and higher culling rate in males (Coelho et al., 2015). *Brucella* has an affinity to gravid uterus and udder in ruminants (Poester et al., 2013; Thompson, 1934).

Age (>4years) and parity status (multiparous) were found significantly associated ($p<0.05$) with higher odds as compared to younger (<4 years) and nulli/primiparous (≤1 parturiated) animals, respectively. Furthermore, age was also found significantly associated ($p<0.05$) with seroprevalence (OR 2.88) by multivariate analysis (Table 3). A similar trend was reported in both sheep and goats with significant association (Ali et al., 2017; Gul et al., 2014), non-significant association (Iqbal et al., 2013) and without determination of association (Ghani et al., 1995; Hussain et al., 2014). This might be due to increased frequency of contact with other animals with respect to age, higher coital chances and sexual maturity as compared to younger animals (Abubakar et al., 2012; Gul and Khan, 2007).

The seropositivity was found significantly associated ($p<0.05$) with Buchi (or Bahawalpuri) breed of sheep kept at Farm 2. Previous reports contradict this result as no statistically significant association could be found in sheep in the district Layyah (Iqbal et al., 2013). A higher seroprevalence was found in crossbred goats (Mirza et al., 1998). Specific genotype might show resistance to the infection, but precise knowledge remains unknown (Coelho et al., 2015). Recently crossbred cows have been found more susceptible to brucellosis infection as compared to local breeds in Pakistan and India (Akhtar et al., 2019; Kumar et al., 2019; Mittal et al., 2018). However, the presence of this specific sheep breed along with bovines might have influenced the results, because spill-over infection is possible in close contact (Afzal and Naqvi, 2004; Assenga et al., 2015; Anonymous).

Reproductive disorders showed significant association (OR 2.69, $p=0.006$) with brucellosis in the current study (Tables 2 and 3). It is understandable as late abortion and retention of foetal membranes are characteristic signs of brucellosis. These findings are supported by similar results reported by others previously (Arshad et al., 2011; Naeem and Kamran, 2013; Khan et al., 2017). However, a non-significant association ($p>0.05$) in sheep has also been reported (Iqbal et al., 2013). Furthermore, animals with ≤3 BCS were found significantly more likely to test positive (OR 12.37, $p<0.001$) in our study. Similar was reported by a study conducted in Ethiopia (Tsegay et al., 2015). A possible reason could be the higher susceptibility of animals already infected with brucellosis to other infections or the loss in BCS caused by the brucellosis itself.

Conclusion

In conclusion, we found anti-*Brucella* antibodies in sheep and goats at these livestock farms in Punjab, Pakistan. Farm location, age and breed of the animals, history of reproductive disorders and BCS were found to play a significant role for brucellosis seropositivity in these animals. Animal species and sex did not seem to play a role here. Although vaccination is recommended and treatment is possible for brucellosis, they are not considered safe for human health, hence regular screening and culling of the reactor animals remain the only choice to

eradicate brucellosis. Introduction of the new stock at these farms should be carried out only after screening and quarantine. Furthermore, farm workers should be advised to adopt protection measures as a routine. Abortion at these farms should not go unnoticed and must be investigated to confirm its cause to adopt recommended control measures. If abortions occur, disinfection of the area should be ensured along with strict biosecurity measures to restrict chances of dissemination of infection through the dogs, cats, other domestic animals, visitors and farm equipment/supply movement. Standardization of the diagnostic tests is required based on the local conditions. Isolation and molecular investigations of the etiological agents might be helpful for future understanding of the epidemiology of the infection and the relationship of the outbreaks.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, Q.U. and T.J.; methodology, F.M. and M.S.; formal analysis, M.H.H.; investigation, T.J. and Q.U.; data curation, U.T., A.I. and Q.U.; writing-original draft preparation, T.J.; writing-review and editing, S.U., Z.I.Q., H.J., S.S. and H.N.

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Permission and ethical approval

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Table 1. Seroprevalence in small ruminants of Punjab, Pakistan

Farm	Goats		Sheep		Total	
	Pos./Tested	Prev.%(95% CI)	Pos./Tested	Prev.%(95% CI)	Pos./Tested	Prev.%(95% CI)
1	0/0	-	0/41	0 (0-8.6)	0/41	0 (0-8.6)
2	0/0	-	18/37	48.7 (31.9-65.6)	18/37	48.7 (31.9-65.6)
3	0/0	-	0/22	0 (0-15.4)	0/22	0 (0-15.4)
4	13/203	6.4 (3.5-10.7)	1/40	2.5 (0.1-13.2)	14/243	5.8 (3.2-9.5)
5	7/44	15.9 (6.6-30.1)	0/88	0 (0-4.1)	7/132	5.3 (2.2-10.6)
6	0/43	0 (0-8.2)	0/9	0 (0-33.6)	0/52	0 (0-6.8)
7	0/0	-	0/45	0 (0-7.9)	0/45	0 (0-7.9)
8	0/0	-	6/145	4.1 (1.5-8.8)	6/145	4.1 (1.5-8.8)
9	6/210	2.9 (1.1-6.1)	0/73	0 (0-4.9)	6/283	2.1 (0.8-4.6)
Total	**26/500**	**5.2 (3.4-7.5)**	**25/500**	**5 (3.3-7.3)**	**51/1,000**	**5.1 (3.8-6.7)**

Table 2. Univariable analysis of the seroprevalence of brucellosis in small ruminants of Punjab, Pakistan

Variable	Category	Pos. / Tested	Prev. % (95% CI)	Odds Ratio	95% CI	p value*
Farm	Farm 2	18/37	48.7 (31.9-65.6)	25.7	12.84-55.52	<0.001
	Others	33/963	3.4 (2.4-4.8)	Ref	-	
Species	Sheep	26/500	5.2 (3.4-7.5)	1.042	0.593-1.831	0.886
	Goats	25/500	5 (3.3-7.3)	Ref	-	
Sex	Females	47/893	5.3 (3.9-6.9)	1.43	0.51-4.05	0.5
	Males	4/107	3.7 (1-9.3)	Ref	-	
Age	Above 4Y	35/440	7.9 (5.6-10.9)	2.94	1.60-5.38	<0.001
	Below 4Y	16/560	2.9 (1.6-4.6)	Ref	-	
Parity Status	Multiparous	40/594	6.7 (4.9-9.1)	2.59	1.31-5.12	0.006
	Nulli/Primi	11/406	2.7 (1.4-4.8)	Ref	-	
Breeds	Buchi	18/37	48.7 (31.9-65.6)	26.7	12.84-55.52	<0.001
	Others	33/963	3.4 (2.4-4.8)	Ref	-	
Reproductive Disorders	Yes	25/178	14.0 (9.3-20.0)	5.00	2.81-8.89	<0.001
	No	26/822	3.2 (2.1-4.6)	Ref	-	
BCS	≤3	34/172	19.8 (14.1-26.5)	11.74	6.39-21.62	<0.001
	>3	17/828	2.1 (1.2-3.3)	Ref	-	

**(Statistical value of significance:* p *value≤0.05)*

Table 3. Multivariable analysis of the seroprevalence of brucellosis in small ruminants of Punjab, Pakistan

Variable	Exposure Variable	Comparison	OR	95%CI	p value*
Farm	Farm 2	Others	34.05	13.47-86.10	<0.001
Age group	>4 years	<4 years	2.88	1.39-5.94	0.004
Reproductive disorders	Yes	No	2.69	1.33-5.42	0.006
BCS	≤3	> 3	12.37	5.98-25.57	<0.001

Model Fit: Nagelkerke R^2 = 0.407, Hosmer and Lemeshow Test (χ^2 = 3.092, p = 0.543)

(Statistical value of significance: p *value≤0.05)*

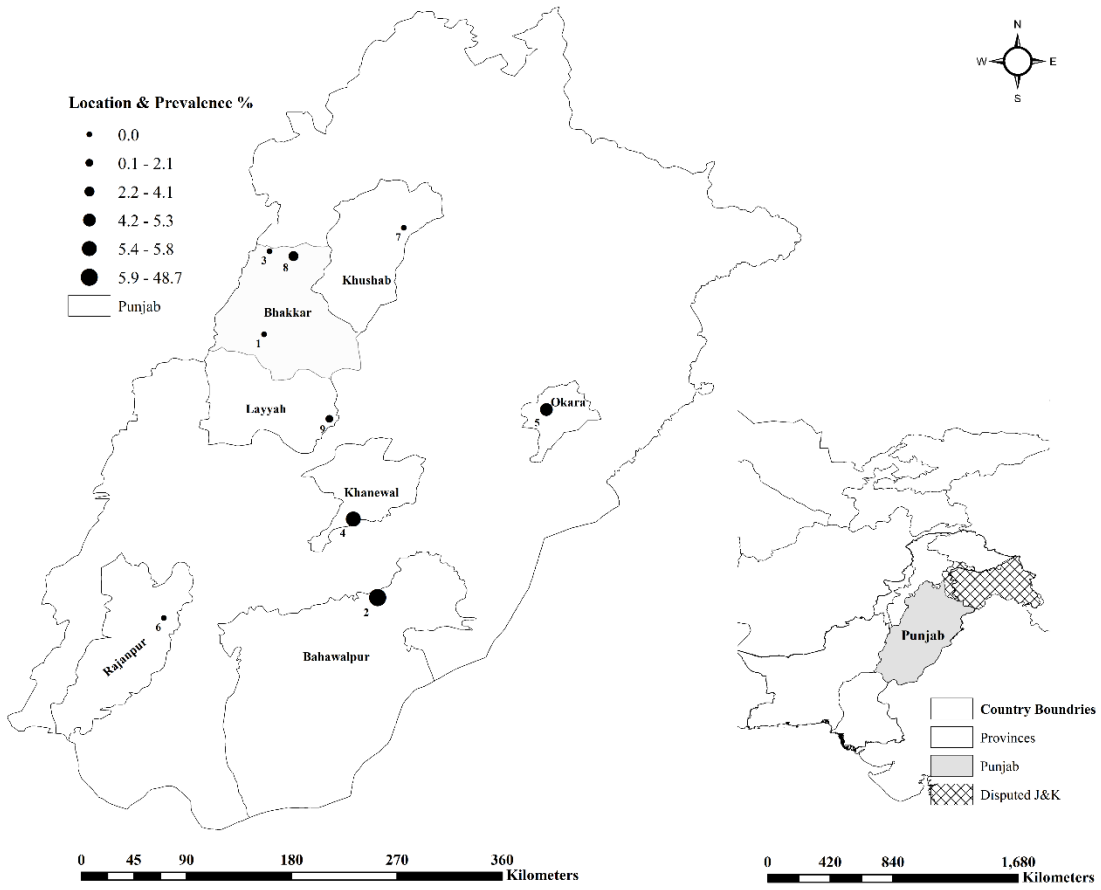

Figure 1. Geographical representation of the small ruminant farms tested for brucellosis in Punjab, Pakistan

4 Discussion

Brucellosis is a zoonotic infection which occurs worldwide, including Pakistan. The main reservoirs for brucellosis are the infected animals disseminating the bacteria with their body secretions. Humans contract the infection either by consumption of contaminated/unpasteurized raw milk or by accidental exposure to infected animals (Abedi et al., 2019; Dadar et al., 2020). In humans, brucellosis is the second most prevalent zoonosis after rabies and reflects the infection status in animals (Abubakar et al., 2012; Bercovich, 1998). It is considered endemic in Pakistan, where reports exist from domestic animals including bovines, small ruminants, camels, equines and recently dogs and humans (Wadood et al., 2009; Ali et al., 2014; Ali et al., 2015; Fatima et al., 2016; Jamil et al., 2019; Saddique et al., 2019; Saleem et al., 2019; Jamil et al., 2020). Pakistan has a significant production of livestock (201.9 millions), milk (59.8 million tons) and meat (4.5 million tons) in the year 2018-2019 where the livestock sub-sector played a share of 60.5% in the agriculture sector and a 11.2% share in the national Gross Domestic Product (GDP) (Anonymous, 2019). Most of the livestock are bovines (87.8 millions) and small ruminants (107 millions) because of dual milk and meat production purposes. Approximately 98% of the milk marketed is raw milk whereas only 2% is processed (Garcia et al., 2003). In this scenario, brucellosis poses a significant threat to public health in the country.

Several non-ruminant domestic animals are suspected in the transmission of brucellosis (Ahmad and Munir, 1995). Moreover, a relative abundant literature was available for “livestock” or “classical” brucellosis (e.g. *B. abortus, B. melitensis* and *B. suis*) but was lacking for non-conventional brucellosis (e.g. *B. canis*) in non-ruminant livestock e.g. dogs which live in close contact to humans as well as domestic ruminant livestock. Hence, the studies were performed using standardized serological and molecular biological diagnostic procedures to identify the possible epidemiological burden and risk factors with suitable statistical analysis.

Serology is the main method for diagnosis of brucellosis in both animals and humans. As anti-smooth-LPS antibodies are the main fraction of antibodies produced by infected livestock animals, most of the diagnostic tests depend on smooth-LPS antigen of approved strains e.g. *B. abortus* strain 99 (OIE, 2018). Biological variables i.e. status of infection, pH of the reagents, antibody conjugates (in ELISAs) etc. may affect the overall performance of the diagnostic tests. RBPT is a cheap, sensitive, and reliable agglutination test for general screening purposes used all over the world (Ducrotoy et al., 2018). ELISA on the other hand was developed to provide robust and specific results with high sensitivity when standardized. It is used often as a secondary test following RBPT. If used as a single diagnostic screening method, standardization and validation are required (Nielsen and Yu, 2010). SAT is another

diagnostic method for brucellosis in humans but is no longer a preferred test in animals for international trade. CFT is a very specific test if used according to the OIE recommendations. Nevertheless, it is complicated and needs standardization and validation under the local conditions along with trained technical staff. Also, the results are dependent on the quality of the sera, anti-complementary activity, and incubation temperature i.e. cold incubation (2-8 °C for 16±2 hours) or warm incubation (37°C for 30 mins). Whatever the diagnostic tests used, every test needs true positive and true negative sera as controls (OIE, 2018). MRT or even the milk indirect-ELISA are efficient and standardized methods for brucellosis screening in lactating cows. However, MRT cannot be used in small ruminants and camels. The major drawback of all diagnostic tests is that they are unable to differentiate anti-smooth-LPS antibodies of field isolates from those of vaccine strains (Nielsen and Yu, 2010; OIE, 2018). In such scenarios, PCR-based diagnostic tests can identify brucellosis precisely if template DNA is present in the sample. Real-time based PCRs are even more sensitive but as the protocols are dependent on the time of sample collection, status of infection, DNA extraction procedure and laboratory conditions, the results are highly variable (Nielsen and Yu, 2010, 2010). Isolation of Brucellae by culture is the gold standard test but is less efficient. The isolates could be further used for a variety of identification and typing tests e.g. Gram staining, biochemical characterization, molecular typing e.g. SNPs, MLST and MLVA typing, which are highly precise modern techniques (Le Flèche et al., 2006; Scholz and Vergnaud, 2013). In wildlife samples, RBPT can be used adequately for brucellosis screening. CFT could be used but complement inactivation temperature and cut-off values need to be properly defined. Both indirect- and competitive-ELISAs provide a better diagnostic solution. However, quality of the sample and validation criteria might be difficult to establish (Godfroid et al., 2010). Isolation of the agent would still be the gold standard in wild animal brucellosis. Brucellin skin test is a useful test based on highly specific cellular immunity in non-vaccinated animals in brucellosis-free areas (OIE, 2018). Nevertheless, all these techniques are resource dependent as they need a lot of technical effort and precise knowledge. Keeping this scenario in mind, it was decided to use RBPT and commercial indirect-ELISA kits for the detection of anti-smooth-LPS antibodies (*B. abortus, B. melitensis* and *B. suis*) and an in-house standardized SAT for the detection of anti-rough-LPS antibodies (*B. canis*) and a validated real-time PCR based detection/differentiation procedure for precise identification of the prevailing brucellae in animals of Pakistan. Previous reports found brucellosis via serology at organized livestock farms but did not identify the etiology precisely (Farooq et al., 2011; Gul et al., 2015; Gul et al., 2014). Hence, we tried to update the brucellosis burden at these farms and identify the etiology precisely.

409 buffalo and 419 cattle sera were sampled randomly from 11 institutional organized livestock farms in Punjab, Pakistan according to the population at each farm. Sera were subjected to commercial RBPT screening followed by indirect-ELISA. An in-house validated

real-time PCR method was followed (Jamil et al., 2020). The overall seroprevalence in buffaloes and cattle by RBPT and indirect-ELISA was 3.9% (32/828) and 3.3% (27/828), respectively. Both tests showed almost perfect agreement (0.877) by Kappa agreement statistic. *B. abortus* was found as the cause of brucellosis at these farms. Buffaloes were found significantly associated ($p<0.05$) and at higher risk (OR 2.71; CI 1.24-5.94) with seropositivity at these farms. The breed was not found to be associated with infection. However, a specific genotype might have influence on the results, but this assumption will need further clarification. Other plausible variables e.g. sex, age and history of reproductive disorders were also not found being associated with seropositivity. Reproductive disorders have been associated with brucellosis in previously published literature from Pakistan (Ali et al., 2017; Ismail et al., 2018; Ullah et al., 2019). Individual animal and farm level variables e.g. sex, size of the herd, husbandry practices and farm biosecurity might have influenced the results in each study. An important reason for the incidence of brucellosis at livestock farms, especially at private farms, is the breach of biosecurity by introduction of new animals for herd replacement or breeding purposes without prior screening. Most of the animals in the animal markets are purchased based on apparent health, but brucellosis can be asymptomatic. These silent carriers pose a high risk to existing brucellosis-free herds at a farm. Owners are advised to seek veterinary health consultation from authorities to be informed on quarantine and biosecurity measures before making a purchase. In Pakistan, biosecurity measures at most of the livestock farms will be basically the physical isolation along with limited personnel access during this time. At institutional livestock farms, biosecurity is practiced at tertiary levels including construction, biocontainment, and routine preventive procedures. Nevertheless, prior screening and quarantine measures would also help avoiding outbreaks. As the institutional farms perform routine screening, brucellosis is commonly reported as abortion storm at private dairy farms. Brucellosis screening antigens, produced by provincial Veterinary Research Institutes (VRIs), for e.g. RBPT, SAT and MRT are available at a fair price for public use. Vaccination is not performed at these institutional farms. As imported elite dairy animals at private farms are of high economic value, the selection of specific vaccines depends on the decision of the owners. The vaccine strain is crucial for the evaluation of the diagnostic results. S19 and RB51 live vaccines are available in Pakistan (Akhtar and Mirza, 1995; Rasool et al., 2018). As S19 vaccine interferes with antibodies produced by field strains, good records of the vaccination practice and reports to the authorities are important. Also, higher biosafety procedures are required while administering these vaccines as they are abortogenic, get secreted into milk and are pathogenic for humans. A systemic review including these variables would be helpful for establishing criteria for the final decision and recommendation of preventive measures to be specifically implemented at these farms.

It was also of interest to know the agreement of the results obtained with RBPT and indirect ELISA. RBPT has been described as an adequate sensitive test for screening of brucellosis worldwide. However, it can react with antibodies against other Gram-negative bacteria e.g. *Yersinia enterocolitica, Salmonella* etc. (Abubakar et al., 2012). ELISAs were developed to address this issue as robust and sensitive diagnostic tests (Uzal et al., 1995). Several commercial kits are available. However, ELISAs need standardization and validation based on the local epidemiological situation. Because of its lower specificity, which is comparable to that of RBPT, standardization requirement and high costs, it is not the best option for screening purposes in resource-limited countries like Pakistan (Jamil et al., 2020). However, development of a locally standardized kit at a fair price would be the best measure. In addition, new highly specific CFT would also need to be standardized and validated according to the disease situation of the area. Based on these results and those of the previously published literature, RBPT appears to be the most adequate test for the current economic and disease scenario of Pakistan.

In order to determine a possible role of non-ruminant domestic animals in the transmission of brucellosis, 181 sera from working and stray dogs were sampled conveniently at two cities in Punjab, Pakistan (87 from Faisalabad and 94 from Bahawalpur). These dogs had close contact to livestock, i.e. bovine and small ruminant herds, and were fed from leftovers from butcheries, slaughterhouses, small livestock farms and household kitchens. I was curious to determine the canine brucellosis burden for which I tested the sera with a SAT by using an in-house standardized *B. canis* antigen as well as livestock brucellosis for which I tested the sera with commercial indirect-ELISA in parallel (Jamil et al., 2019). The seropositive sera were then subjected to DNA extraction by a commercial DNA extraction kit followed by a genus- and species-specific in-house validated real-time PCR (Probert et al., 2004; Gwida et al., 2012). I found 37.6% (68/181) of the sera being positive by SAT for canine brucellosis (*B. canis*) and 4.9% (9/181) being positive by indirect-ELISA for livestock brucellosis (*B. melitensis* and *B. abortus*). *B. abortus* DNA was amplified from one indirect-ELISA-positive serum. Location (Bahawalpur) and age (1-2 years) were found to be significantly associated ($p<0.05$) with canine brucellosis (*B. canis*). Presence of wounds was found to be significantly associated and a higher risk for livestock brucellosis (*B. abortus*), probably indicating accidental exposure during competition for food. A previous study was limited to detect only anti-smooth-LPS antibodies in dogs (Ahmad and Munir, 1995). Our study was the first-ever to report *B. canis* and *B. abortus* in dogs in Pakistan. These findings indicated that the dogs, when in close contact to livestock, can transmit brucellosis to other domestic animals and humans. These findings also highlight the importance to include non-ruminant domestic animals in close contact to ruminants in brucellosis eradication programs (Bercovich, 1998; Wareth et al., 2017). In addition, dogs might transmit stealthy zoonotic canine brucellosis (*B. canis*).

For small ruminants, 500 sheep and 500 goat sera were randomly sampled from nine different institutional organized farms considering the population proportions and the upper possible limits of sampling procedure. Screening by commercial indirect ELISA was followed by DNA extraction and application of validated in-house real-time PCR (Gwida et al., 2012). Overall, a 5.1% (51/1000) seroprevalence was found with almost identical rates in sheep (5.0%;25/500) and goats (5.1%; 26/500). *Brucella*-DNA was not detected by real-time PCR in any of the tested sera. Host species was not found statistically significant for sheep and goats. However, the farm location had a significant association ($p<0.05$) with seropositivity. Age, parity status, breed of the animal, history of reproductive disorders and body condition score were also significantly associated ($p<0.05$) with seropositivity whereas the sex of the animal was not found to be associated with seropositivity. In pregnant ruminants, *Brucella* has an affinity to the gravid uterus, the reproductive tract, and the udder. This might have influenced the results when compared with males and non-pregnant animals. As in my previous study, individual animal and farm level variables might have had an influence on the results (Jamil et al., 2020). Now, a systemic review including all variables studied is needed to understand the influence of these variables not only at farm level, but also for the general animal population.

Our studies, in the light of previously published reports, indicate that the main cause of brucellosis in livestock and humans in Pakistan is *B. abortus* and to some extent *B. melitensis*. Both have been isolated from bovines and goats, respectively, and have been typed precisely by MLVA-16 typing (Ali et al., 2019; Mahmood et al., 2016). However, studies describing SNPs and MLST (especially the cgMLST) based on whole genome sequencing are lacking. Thus, there is a need of further work on brucellae in the country as well as a need for culturing of these bacteria as molecular studies still require high quality DNA, which is best obtained from cultured isolates. In addition, this amendment will need establishing and maintaining biosafety standard operation practices for the existing labs as well as upgrading of the existing ones. Another main task will be training of the laboratory staff on how to reduce and avoid infection risks for the technicians. MALDI-TOF including high throughput sequencing technologies have drastically reduced the time for identification and typing of *Brucella* spp. in developed countries. A combination of these technologies would improve counter measures in human and animal medicine in Pakistan as well, but high initial and running costs will keep hindering the introduction of these techniques in developing countries. Molecular typing can help to identify the epidemiological origin and the genetic relationships of the isolates in an outbreak to timely identify and interrupt the infection chains.

There has been no report of *B. suis* in the country to date and *B. suis* was considered not to be present in the area (Mohydin, 1979). Reports on canine brucellosis (*B. canis*) in humans are also lacking. However, further studies are needed to elucidate the real situation for Pakistan. Further on domestic non-ruminant animals e.g. equines as well as wildlife need

further investigations to reveal their possible role in brucellosis transmission. Although ectoparasites have not been associated with the transmission of brucellosis in Pakistan, a report does exist (Wang et al., 2018). This finding needs further evaluation especially isolation of brucellae from these pests.

To conclude, brucellosis remains a challenging zoonosis especially in endemic areas of Pakistan. A low level of anti-*Brucella* antibodies should be expected in ruminants at any time at any case if the disease has not been eradicated but is only controlled. Vaccination is limited and is not widely accepted based on safety issues. Treatment could be a useful alternative but so far no 100% safe cure is available and further studies are required to develop a suitable regimen. Normally, test and slaughter policy could be recommended at a herd prevalence of ≤2% for a brucellosis eradication program. However, this procedure is no option for small farmers in developing countries. A safe vaccine for humans needs to be developed to protect professional personnel in the animal production of endemic areas. Food hygiene shall reduce the risk for consumer. Although Veterinary Research Institutes (VRIs) are producing and standardizing biologicals already in Pakistan, reference laboratories are needed to serve for the new control programs to be implemented by the government in the near future, which will include vaccination of the food producing ruminants. Besides vaccination, the following measures are recommended:

1. Non-ruminant domestic animals and camels should not be ignored in national brucellosis eradication programs.
2. Quarantine measures should be strictly implemented when purchasing new animals or moving/receiving animals to new areas.
3. The milk chain needs to be controlled.
4. Medical authorities are needed to be involved to tailor an eradication program in an “One Health” approach, including the education of physicians and consumers.
5. Awareness programs for farmers and education along the animal production chain should be implemented.
6. Monitoring of *B. canis* and *B. ovis* infection in animals and canine brucellosis in humans is required.
7. Replacement of chronically infected animals is necessary and the compensation of animal losses e.g. by private and public financed insurances shall be established.

5. Summary

Diagnosis and molecular biology of *Brucella abortus* in Pakistan

Brucellosis is a zoonotic disease worldwide and remains a persistent problem in domestic ruminants in Pakistan. It is mainly caused by *B. abortus* and to some extent by *B. melitensis*. In humans, it is related to either professionals after accidental exposure or to the general public via consumption of contaminated unpasteurized milk. Brucellosis vaccination is rarely practiced in the ruminants of the country, yet. Although the scientific community is engaged in brucellosis research, animal holders, veterinarians and physicians are not aware of the disease and the zoonotic risk posed by infected animals.

A total of 828 bovine (409 buffaloes and 419 cattle), 1,000 small ruminant (500 sheep and 500 goats) and 181 dog sera were collected from organized livestock farms and stray and working dogs in close contact to ruminants from various locations across Punjab, Pakistan, respectively. The sera were subjected to RBPT, iELISA (for *B. abortus* and *B. melitensis*) and SAT (for *B. canis*) and genus- (*Brucella*) and species-specific (*B. abortus* and *B. melitensis*) real-time PCR. In bovines, an overall seroprevalence of 3.9% was found and *B. abortus* was identified as the etiological agent. In small ruminants, a prevalence of 5.1% was found but the causative bacteria could not be identified, as all investigated sera were negative by real-time PCR. A total of 4.9% of dogs had livestock brucellosis (*B. abortus*) confirmed by real-time PCR, whereas 37.6% were positive for canine brucellosis (*B. canis*) using SAT.

Risk factors identified for bovines were location and species (buffaloes), for small ruminants location, age (>4years), parity status (>1), breed of the animal and reproductive disorders and for dogs location, age (≥1 years) and a weak body condition (for *B. canis* infection) and the presence of wounds (for *B. abortus* infection). These risk factors were significantly associated with brucellosis with higher risk odds. Thus, location and age were the most associated risk factors in all studies.

It was shown that brucellosis is a persisting problem at organized livestock farms in Punjab, Pakistan. *B. abortus* is the disease-causing agent. An alarmingly high prevalence of canine brucellosis caused by *B. canis* was detected. Robust, cheaper, and more reliable diagnostic tests are needed for both, smooth and rough-LPS *Brucella* spp., to meet the needs of the local situation. Isolation of the bacteria at any case will be needed for preparation of high-quality DNA for advanced molecular and comparative epidemiological studies. Nevertheless, advanced training, knowledge about the disease dissemination, biosafety and biosecurity measures, awareness programs and implementation of these measures at farms and laboratory levels are obvious needs to combat brucellosis in the future in Pakistan.

6 Zusammenfassung

Diagnose und Molekularbiologie von *Brucella abortus* in Pakistan

Die Brucellose ist eine weltweit auftretende Zoonose und bleibt ein anhaltendes Problem bei Hauswiederkäuern in Pakistan. Sie wird hauptsächlich von *B. abortus* und teilweise von *B. melitensis* verursacht. Beim Menschen besteht ein Zusammenhang mit beruflicher Exposition oder dem Konsumieren von kontaminierter, nicht pasteurisierter Milch. Brucellose-Impfungen werden bei Wiederkäuern in Pakistan noch selten durchgeführt. Obwohl sich die wissenschaftliche Gemeinschaft intensiv mit Brucelloseforschung befasst, sind sich Bauern, Ärzte und Tierärzte der Krankheit und des zoonotischen Risikos infizierter Tiere nicht bewusst.

Insgesamt wurden 828 Seren von Büffeln (409) und Rindern (419), 1000 Seren kleiner Wiederkäuer (500 Schafe und 500 Ziegen) und 181 Hundeseren von organisierten Tierfarmen bzw. von streunenden Hunden und Arbeitshunden, die in engem Kontakt mit Wiederkäuern standen, aus verschiedenen Orten im Punjab, Pakistan gesammelt. Die Seren wurden mit RBPT, iELISA (für *B. abortus* und *B. melitensis*) und SAT (für *B. canis*) untersucht und gattungs-(*Brucella*) sowie speziesspezifischer (*B. abortus* und *B. melitensis*) real-time PCR unterzogen. Bei Rindern wurde eine Gesamtseroprävalenz von 3,9% gefunden und *B. abortus* als ursächlich beteiligtes Bakterium identifiziert. Bei kleinen Wiederkäuern betrug die Prävalenz 5,1%, aber die beteiligte *Brucella* spp. konnte nicht identifiziert werden, da alle untersuchten Seren in der real-time PCR negativ waren. Hunde litten zu 4,9% an Wiederkäuerbrucellose (*B. abortus*), was durch die real-time PCR bestätigt wurde. Insgesamt 37,6% der mittels SAT untersuchten Hunde waren positiv für Hunde-Brucellose (*B. canis*).

Die für Rinder identifizierten Risikofaktoren waren Standort und Spezies (Büffel); für kleine Wiederkäuer Standort, Alter (>4 Jahre), Gebährstatus (>1), Tierrasse und Fortpflanzungsstörungen sowie für Hunde Standort, Alter (≥1 Jahre) und schwacher Körperzustand für *B. canis*-Infektionen und das Vorhandensein von Wunden für *B. abortus*-Infektionen. Diese Risikofaktoren waren signifikant mit höheren Odds-ratio Werten für Brucellose korreliert. Standort und Alter waren in allen Studien die häufigsten Risikofaktoren.

Im Rahmen der vorliegenden Studie hat sich gezeigt, dass Brucellose in organisierten Tierfarmen im Punjab, Pakistan ein anhaltendes Problem darstellt. *B. abortus* ist dabei der am häufigsten identifizierten Erreger. Eine alarmierend hohe Prävalenz von Hunde-Brucellose verursacht durch *B. canis* wurde festgestellt. Robuste, billigere und zuverlässigere Diagnosetests sind sowohl für Brucellen mit glattem und rauem LPS erforderlich, um den Anforderungen der örtlichen Situation gerecht zu werden. Die Anzüchtung der Brucellen ist in jedem Fall erforderlich, um DNA von hoher Qualität für weitere molekulare und vergleichende epidemiologische Studien zu gewinnen. Schulungen und andere Maßnahmen zur Verbreitung von Wissen über die Krankheit, Maßnahmen zur Vermittlung von Grundlagen der biologischen

Sicherheit, Aufklärungsprogramme und die Umsetzung dieser Maßnahmen in landwirtschaftlichen Betrieben und auf Laborebene sind unabdingbare Voraussetzungen für eine effiziente künftige Bekämpfung der Brucellose in Pakistan.

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9 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegeben Quellen und Hilfen in Anspruch genommen habe.

Jena, den 30.09.2020 **Tariq Jamil**

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