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Serological and molecular coverage of brucellosis in high-risk human population of district Swabi, Khyber Pakhtunkhwa, Pakistan

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Abstract

Brucellosis is a contagious and zoonotic disease, affecting human and animals equivocally. It is a seriously neglected disease in underdeveloped countries, causing heavy financial losses in the livestock sector in terms of abortion, and a decrease in milk production. To better cope with socioeconomic impact of brucellosis, this study was conducted in district Swabi with the aim to detect brucellosis in high-risk human population through serological Rose Bengal plate test, Serum plate agglutination test, Enzyme-Linked Immunosorbent Assay, and molecular Polymerase chain rection techniques with respect to different risk factors and clinical history. A total of 250 blood samples (n=250 each from Human) were collected through predesigned questionnaire and were processed for detection of brucellosis through different techniques in Veterinary Research Institute, Peshawar. Detection of brucellosis frequency was performed through SPAT, RBPT, Indirect ELISA (IgM, IgG) and PCR as 12.4, 10.4, 11.6, 12 and 9.6% respectively. A numerical difference was observed through detection of different tools, but the difference was statistically non-significant ($p>0.05$). The present research contributes to the existing prevalence data concerning brucella infection in humans and emphasizes the benefits and effectiveness of the molecular technique of PCR compared to serological tests.

Keywords: Bovine brucellosis, SPAT, RPBT, ELISA, PCR, Human, Swabi

Introduction

Brucellosis is a contagious and infectious zoonotic disease, which infects several mammalian species, including humans, and some amphibians except cats, which is naturally resistant to brucellosis (Alp et al., 2008). In humans, this disease is known by multi names i.e. Malta fever, Rock fever of Gibraltar and mostly by undulant fever. The incubation period varies from 1 week to months. The symptoms include fever, general malady, night sweats, weaknesses, body pain and joints pain. It is a multi-systemic disease that can cause neuro-brucellosis which is manifested by meningitis, encephalitis, stroke, radiculitis, myelitis and neuropsychiatric complications (Gunduz et al., 2017; Dias et al., 2018). It is a socioeconomic disease causing heavy economic losses globally, in term of abortion and decrease in milk production, especially in developing world where most of the people depend on livestock for their livelihood (Barbier et al., 2011).

Sir David Bruce in 1887, first time demonstrated brucella organism by processing spleen specimen collected from soldier died due to febrile fever on the Island of Malta, later the organism was termed as Micrococcus melitensis. In 1897, Bernard Bang isolated Bacillus abortus in cattle, causing abortion in cattle and undulant fever in man (Kunkle et al., 1995; Cutler et al., 2005). Brucella is a gram-negative, non-motile, anaerobic, coccobacilli and facultative intracellular bacteria, belongs to the order Rhizobials and class α- proteobacteria (Gupta et al., 2005). Brucella has 6 species, each having specific biovars. Brucella melitensis has 1–3 biovars, infecting ovine, caprine, bovine and human. Brucella abortus having 1–6 and 9 biovars cause disease in bovine and human. In the same way, the Brucella suis 1–3 biovars cause disease in swine. Brucella canis infects canine, Brucella ovis in ovine and Brucella neotome in desert wood rats. Brucella pinnipedialis and Brucella ceti are recently known to cause disease in oceanic mammals (Foster et al., 2007). B. abortus, B. melitensis and B. suis were regarded as primary etiological agents of brucellosis in third world poor countries (Corbel, 1997). Brucella genome has two chromosomes, lacking plasmids, which is a distinctive property in the bacteriacea family (Del Vecchio et al., 2002).

Brucellosis is one of the utmost extensive zoonosis, triggering substantial sickness in animals and humans across the globe. Among the zoonotic bacterial infections, brucellosis is on top ranking, with about 0.5 million cases recorded annually in endemic areas (Moreno et al., 2014; Khan et al., 2018). Brucellosis is transmitted between animals and human through aerosol, raw milk, semi-cooked meat products or direct contact with infected animals (Gwida et al., 2010). High risk individuals (livestock farmers, laboratory workers, veterinarians, butchers and hunter) are more likely to acquire the disease by direct contact with an animal or through infective tissue, accidental pricks, cuts or abrasions and through inhalation (Ayylidiz., 2007). Brucellosis is easily acquired laboratory infectious disease due to the aerosol transmission and low infective dose (Pardon et al., 1978; Weinstein et al., 2009; Mense et al., 2004). Brucellosis is a widespread disease in many Asian countries i.e. India, China, Sri Lanka and Pakistan, both in humans and livestock. In Pakistan, sero- prevalence was found to be 21.7% through ELISA while molecular findings by real-time PCR were recorded as 6.9% in human (Norman et al., 2016; Wang et al., 2017). In another study, the occurrence in animals and herd level was reported as 6.3 and 18.6%, respectively (Ali et al., 2017). Recently, the prevalence of brucellosis was recorded as 18% in animals and 23% in human by plate agglutination test (SPAT) in Swat area (Saif et al., 2019). Accurate diagnosis of brucellosis is a milestone for limitation and eradication of said zoonosis.

Brucellosis is normally diagnosed through clinical signs/history i.e. upon the occurrence of abortion storm, retention of placenta, still birth, orchitis, and epididymitis in livestock, but these findings may be plausible and must be supported by laboratory findings. At present, there is no single exact technique to detect the causative agent. Culture from blood or bone marrow was considered as “gold standard” with some constraints i.e. time consuming (5 days up to several weeks) and chances of laboratory acquired

illness. The routine test used for screening and diagnosis is serology because these techniques are easy to perform and proved to be safe for laboratory workers (Bricker et al., 2002). Different serological tests are used currently for the screening of brucellosis, but each test has its own limitations (OIE, 2008). At the movement, there is no single nominated antibody which we considered as a reference for diagnosis, therefore, a combination of several techniques is in practice. Serological techniques used for the diagnosis of brucellosis are Serum plate agglutination test (SPAT), Rose Bengal plate test (RBPT), Complement fixation test (CFT), and Enzyme-linked immune sorbent assay (ELISA) but these might result in false-positive results because of cross-reactivity (Arabaci et al., 2012). Serological examination and culturing of organism required specialized bio-containment facilities for limiting laboratory acquired infections. Along with serological techniques, different molecular tests i.e. PCR, are also used for detection of brucellosis and are mostly used in combination with serological tools for detection of brucellosis (Gwida et al., 2011). PCR is used mostly for confirmation of brucellosis positivity as it guarantees high specificity and sensitivity (Weinstein et al., 2009). Due to variation in the level of detection through different diagnostic techniques, the current study was designed for detection of brucellosis through serological (SPAT, RBPT, ELISA) and molecular tests (PCR) in high-risk human population in district Swabi. The map of Swabi district showed in the (Figure. 1), of Khyber Pakhtunkhwa.

Materials and Methods

Study area

The current study was conducted in Veterinary Research Institute, Peshawar and samples were collected from district Swabi. The latitude and longitude of Swabi district is 34.1442 °N and 72.3785 °E and spread over an area of 1543Km^2. District Swabi is divided into four administrative units (Tehsils) like Swabi, Topi, Razzar, and Lahore. The altitude of the district above the sea level ranges from 316-360 m, with an average rainfall of 590 mm. Over the year, temperature ranges from 9˚C to 44˚C, and its climate falls into a humid subtropical zone. The estimated population of the district is 16.8 million heads.

Sampling area

The study was carried out in four, Tehsil of District Swabi. Detail information mentioned in pre-planned questioner was filled on spot to better understand the associated practices which leads to brucellosis in closely related human population i.e. Livestock farmer (male and female, veterinary field staff, butchers, laboratory worker, female home involve in animal keeping at home. The samples were collected from Tehsil Topi, Swabi, Lahore, and Razzar, and transported through the cold chain to Brucellosis Section, Veterinary Research Institute, Peshawar for further processing.

Collection of Samples

A total of 250 whole blood specimens (250 human) in Ethylene diamine tetra acetic acid (EDTA) and Gel clot activator tubes were collected from high-risk human professionals (animal keeper, Shepard's, Butchers, Veterinary practitioners) at different Tehsils of District Swabi. Blood was collected aseptically by venipuncture in humans' blood was collected from a cephalic vein. All the samples were properly marked and labeled accordingly and were transported to Veterinary Research Institute, Peshawar. Serum was harvested by centrifuging blood samples at 4000 rpm in 10 minutes. All the samples were stored in the refrigerator till further processing.

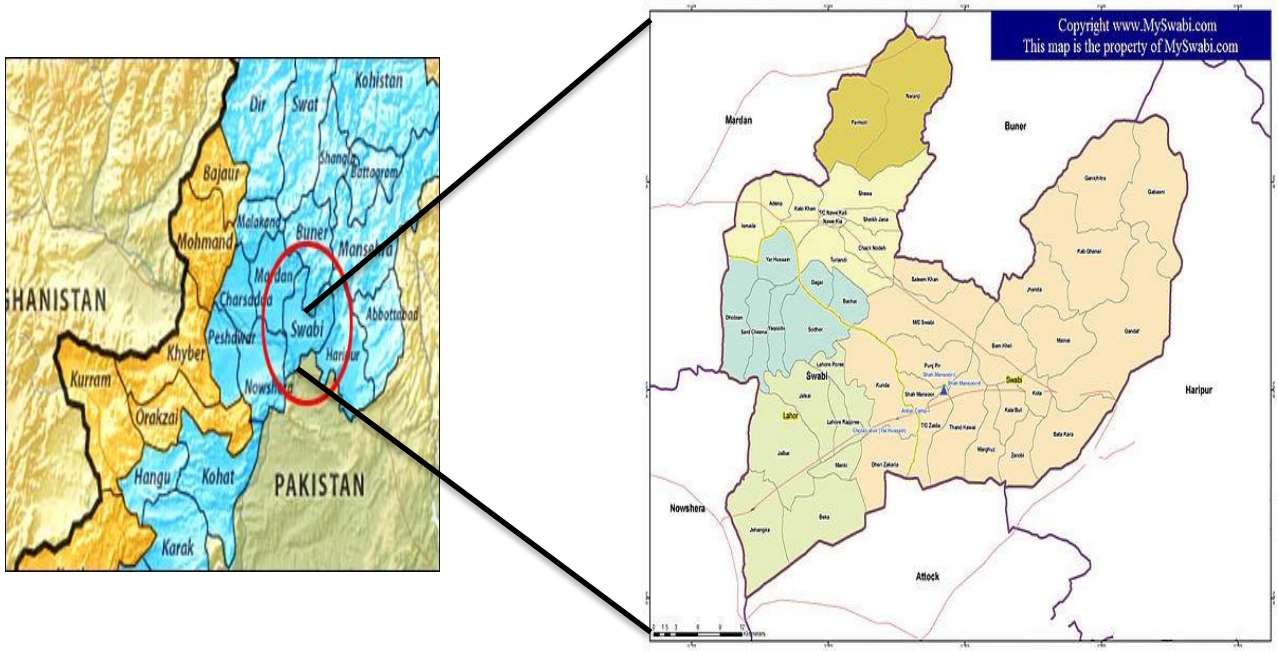

Figure.1. Map of the District Swabi Khyber Pakhtunkhwa

Serological Test

Rose Bengal Precipitation Test (RBPT)

The Rose Bengal test was conducted as described by Alton et al. (1988). The Rose Bengal-stained antigen Lilli dale UK and serum samples were thawed to room temperature. After properly thawing, an equal amount of antigen and serum were mixed on a clean glass slide through sterilized stirrer. Precipitation was observed after five minutes in positive samples while negative samples showed no precipitation.

Standard Plate Agglutination Test (SPAT)

The standard plate agglutination test was conducted according to Alton et al., (1988). Briefly, a clean glass plate was divided into 1.5 square inches. The micro-pipette was used to take different concentration of serum and dropped on glass plates from left to rightward in designated squares i.e. 80, 40, 20, 10, and 5 µl volume. The positive control of the known titter and negative control serum was also added to the last two squares on the glass plate. One drop of both antigen (B. abortus and B. melitensis) suspensions was put on each quantity of serum in squares. The mixture was stirred with a sterilized applicator stick. For each square a separate applicator was used. The glass slide was rotated in the clockwise and anticlockwise direction for 2-3 minutes and the final reading was recorded after 6 minutes. Agglutination was observed by slanting the plate over the excellent light source to visualize any degree of precipitation in square area. There was different concentration of serum which represented as 1:20, 1:40, 1:80, 1:160 and 1:320 dilutions. The titter of 1:80 or above was considered as positive, 1:40 was regarded as doubtful, and 1:20 was declared as negative samples.

Indirect ELISA

For detection of IgM and IgG antibodies in human serum (Vircell Microbiologists Spain, with catalog Numbers MI 006 and GI 003) indirect ELISA kit was used according to manual provided with kits. ID (Innovative Diagnostics) screen Brucellosis serum indirect, France, (catalog No. BRUS-MS-5P) ELISA kit was utilized for cattle serum, according to manufacturer instructions (Fadeel et al., 2011).

IgM Assay Procedure

All reagents of the kit were brought to room temperature and wash solution (20×) was diluted to 1×, by adding 95ml of distilled water to 5ml of 20×wash solution for making of 100ml working solution. Four wells were assigned as controls i.e. one for positive control, one for negative control and two wells for the cut off serum. IgM ELISA for human serum samples was conducted as per directions of manufacturer. Firstly, 25 µl of IgG sorbent was added to designated wells/strips and 5 µl of serum samples were added followed by 75 µl of serum diluent. Similarly, 100 µl of diluent for serum was added to four control wells and for uniform mixing of reagents, plate was shaken for two minutes. The plate was covered with sheets and kept for incubation at 38ºC for 45 min. After completion of incubation, each well was washed 5 times with 300 µl of washing solution. Conjugate solution (100 µl) was added to each well and after covering the plate with aluminum foil, the plate was incubated at 38ºC for 30 min. Each well was washed 5 times again with 300 µl of washing solution. Substrate solution (100 µl) was added to each well and plate was incubated at room temperature for 20 min. Stop solution (50 µl) was added to each well to stop the reaction and optical density (O.D) was recorded through ELISA reader (AMP Platos RII) at 450/620nm.

Result interpretation:

Mean cut off serum OD values were calculated, and Antibody index was calculated according to the formula.

$$\text{Antibody Index} = \frac{\text{Sample OD}}{\text{Cut off serum mean OD}} \times 10$$

Serum samples with index value below 9 were considered as negative, index values with 9-11 were considered as equivocal and index value above 11 was interpreted as positive for IgM antibodies against brucella.

IgG Assay Procedure:

All reagents of the kit were brought to room temperature and wash solution (20×) was diluted to 1X. Four wells were assigned as controls i.e. Positive control, Negative control and two wells for Cut off serum. IgG ELISA for human serum samples were conducted as per directed by manufacturer. Add 100 µl of serum diluent to all wells followed by addition of 5 µl of serum samples and four controls in corresponding wells, for uniform mixing of reagents, plate was shacked for two minutes. Covered the plate again and kept for incubation at 38ºC for 45 min. After completion of incubation, each well was washed five times with 300 µl of washing solution. Immediately (100 µl) IgG conjugate solution was added to each well and after covering the plate with sealing sheet, the plate was incubated at 38ºC for 30 min. Each well was washed 5 times again with 300 µl of washing solution. Substrate solution (100 µl) was added to each well and the plate was incubated at room temperature for 20 min. Stop solution (50 µl) was added to each well to stop the reaction and OD was recorded through ELISA reader (AMP Platos RII) at 450/620nm. The result was calculated and interpreted as done in IgM ELISA.

Result interpretation

Mean cut off serum OD values were obtained, and Antibody index was calculated according to the formula.

$$\text{Antibody index} = \frac{\text{sample OD}}{\text{Cut off serum mean OD}} \times 10$$

Serum samples having index value below 9 were considered as negative, indexes value of 9-11 equivocal and indexes above 11 were considered as positive for IgG antibodies against Brucella.

Interpretation of Results

Calculation of S/p percentage (s/p %) was done by utilizing control and sample OD values as follows.

$$S/P\% = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}} \times 100$$

Results were concluded according to s/p % value as below:

s/p % Value	Status
≤ 110 %	Negative
110-120%	Doubtful
≥ 120%	Positive

Polymerase Chain Reaction (PCR)

DNA extraction

Nucleic acid was extracted from whole blood through Genomic DNA Tissue kit (Nucleo-Spin Tissue, Germany) according to the manufacturer's instructions. Briefly, Proteinase K (25µl) and 200µl blood were added in Eppendorf tube and vortex for homogenous mixing. Buffer B3 (200µl) was added to the tube and vortex again for 15-25 seconds. The tubes were then incubated at 70ºC for 25 minutes to ensure proper lysis. After incubation absolute ethanol (210µl) was added for the binding of DNA, the contents were poured into tissue column placed in a collection tube. The filled columns were centrifuged at 11000 rpm for 1 min and the liquid drained into collection tube was discarded and nucleospin column placed in a new collection tube. For washing of column, 500µl of Buffer BW was added to the column and centrifuged at 11000 rpm for one minute. Flow through was discarded. The column was washed again with 600µl of Buffer B5 and centrifuged again to remove the ethanol residues. Elution buffer (100µl) was incorporated in the column and incubated for 5 minutes at room temperature for and centrifuged at 11000 rpm for one minute. DNA was collected in a collection tube and was ready to utilize in PCR reaction.

Quantification of DNA

DNA extracted was checked for purity and concentration through Nanodrop (Thermo Fisher). The concentration of extracted DNA was in the range of 40-100 ng/µl and the purity (A260/280) was in the range of 1.5-1.7.

Primers used in the study

For identification of genus of Brucella, BCSP 31 gene was targeted with following primers having amplicon size of 223bp (Ali et al., 2013).

B4 forward: 5'- TGGCTCGGT TGCCAATATCAA-3'
B5 reverse: 5'- CGCGCTTGCCTTTCAGGTCTG-3'

PCR reaction

A total of 25µl PCR reaction mixture was prepared by mixing 4 µl master mix (Solis biodyne), 1µl of both forward and reverse primers (10pmol), 16 µl of PCR water and 3µl of template DNA.

Reaction conditions for PCR reaction was.

Initial denaturation	94ºC for 4 min	
Cyclic denaturation	93ºC for 30sec	35 cycles
Annealing	57 ºC for 30 sec	
Cyclic extension	72 ºC for 30 sec	
Final extension	72 ºC for 10 min	

Gel electrophoresis and documentation

After PCR amplification, the PCR product was subjected to gel electrophoresis (Hotzel et al., 1996). For gel electrophoresis, 1% gel was made by mixing 1gm of agarose in 100 ml TBE buffer (1x) in a conical flask. The solution was boiled in microwave oven for 3 minutes and was cooled up to 55 ºC for the addition of ethidium bromide (0.4 µl). The solution was poured in gel caster, and the comb was applied for well formation.

After solidification of the gel, 7µl of DNA ladder (100bp) and PCR product (10µl) was added to the designated wells. The gel was placed in a gel electrophoresis tray having 1x TBE with voltage (110V), current (500mA) and time (30min). After completion of electrophoresis, the gel was placed in a gel documentation system for visualization of the bands of the brucella genus at 223bp (Baily et al., 1992).

Statistical analysis

During sampling, data was collected from the respondents on the predesigned questionnaires and recorded data from questionnaires was compiled in Microsoft excel spread sheet. The occurrences of brucellosis in association with different factors were analyzed through Chi-square test (χ^2) by SPSS (21.00) for the determination of significance at a probability level ≤ 0.05.

Results

Detection of brucellosis in high-risk human population

Human blood specimens were screened by SPAT, RBPT, i-ELISA (IgM & IgG) and PCR. Among 250 specimens, 12.4, 10.4, 11.6, 12 and 9.6% cases were detected positive through SPAT, RBPT, IgM ELISA, IgG ELISA and PCR, respectively. Which showed in the (Figure.2). A numerical variation was observed in results by applying various techniques, but the difference was statistically non-significant (P>0.05) (Table 1).

Table 1. Overall detection of brucellosis in humans

Test Performed	Total samples	Positive samples (n)	Positive percentage (%)	P value
SPAT	250	31	12.4	0.82
RBPT	250	26	10.4	
IgM ELISA	250	29	11.6	
IgG ELISA	250	30	12	
PCR	250	24	9.6	

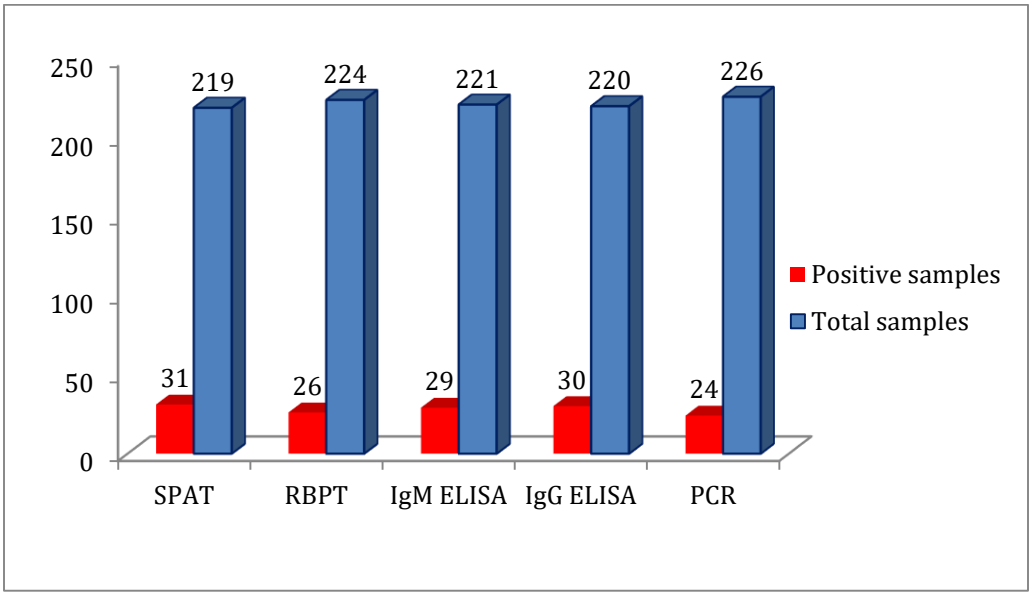

Figure.2. Human samples positive for brucellosis through SPAT, RBPT, ELISA (IgM, IgG) and PCR

Detection of brucellosis in high-risk human population through SPAT

Brucellosis was detected in high-risk human population in different tehsils of district Swabi through Serum plate agglutination test (SPAT) and its association with location, age, gender and professions of individuals and showed in the (Figure.3). A significant difference ($P<0.05$) in the occurrence of brucellosis was recorded, with a higher number of positive cases in tehsil Lahore (20.34%), followed by Razzar (19.4%), Swabi (7.02%) and Topi (1.59%). Age and gender-wise occurrence of brucellosis were found to be non-significant. A numerical difference was observed in the occurrence of brucellosis in different age groups, with a high number of cases that were detected in age group 30-45 years (14.28%), followed by 15-30 years age group (12.6%), more than 45 years age group (8.33%) and least number of cases in age group less than 15 years (5.88%). Sex-wise occurrence of brucellosis was detected higher in female (15.9%) than in male animals (11.59%) and Profession wise data discovered that, the occurrence was higher in field veterinarians (24.48%), followed by housewives (21.1%), butchers (15%), livestock farmers (10.86%), farm workers (8.33%) and researchers (2.22%). The difference in the occurrence of brucellosis among different professions was statistically significant ($P<0.05$) (Table 2).

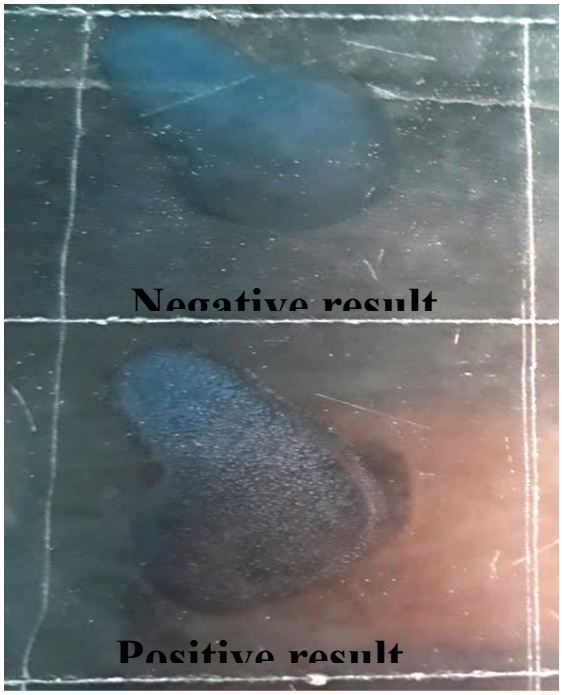

Figure.3. Photograph showing positive and negative results of brucellosis through SPAT in human

Table 2. Detection of brucellosis in human and its association with location, age, sex and professions through SPAT

Variables	Categories	Total sample (n=250)	Positive samples (n)	Positive percentage (%)	P-value
Location	Swabi	57	4	7.02	0.00*
	Topi	63	1	1.59	
	Lahore	59	12	20.34	
	Razzar	72	14	19.44	
Age	<15Y	17	1	5.88	0.72
	15-30Y	119	15	12.60	
	30-45Y	91	13	14.28	
	>45Y	24	2	8.33	
Sex	Female	44	7	15.90	0.43
	Male	207	24	11.59	
Profession	Field vets	49	12	24.48	0.01*
	Farm workers	71	6	8.33	
	Livestock farmers	46	5	10.86	
	Butchers	20	3	15	
	Housewives	19	4	21.1	
	Researchers	45	1	2.22	

Detection of brucellosis in human and its Association with different Factors by SPAT

Detection of brucellosis in human by SPAT, and its association with different factors and clinical history were described in (Table 3). Raw milk consumption was found to be statistically significant (P<0.05) with the occurrence of brucellosis. The number of cases was observed in people who consumed raw milk (32%) as compared to those who did not consume raw milk (10.1%). Similarly, the use of PPEs was significantly (P<0.05) associated with the occurrence of brucellosis. Fewer no. cases were observed in people using PPEs (3.7%) as compared to those who were not using PPEs (18.7%). Among clinical history, fever and backache were found significantly (P<0.05) associated with the occurrence of brucellosis through SPAT test. The occurrence of brucellosis was higher in those having fever (21.6%) as compared to those having no fever (9.4%) and backache was observed in 40% cases having brucellosis as compared to those having no backache (10.5%). However, non-significance (P>0.05) was observed for other clinical signs i.e. joint pain, body pain, night sweats and headache through SPAT.

Table 3. Serological detection of brucellosis in humans through SPAT and its association with different factors

Variables	Categories	Total sample (n=250)	Positive samples (n)	Positive percentage (%)	P-value
Raw milk consumption	Yes	25	8	32	0.00*
	No	226	23	10.1	
PPEs	Yes	107	4	3.7	0.00*
	No	144	27	18.7	
Fever	Yes	60	13	21.6	0.01*
	No	191	18	9.4	
Joint pain	Yes	42	7	16.6	0.36
	No	207	24	11.5	
Body pain	Yes	46	8	17.3	0.25
	No	205	23	11.2	
Night sweats	Yes	19	4	21	0.23
	No	232	27	11.6	
Backache	Yes	15	6	40	0.00*
	No	236	25	10.5	
Headache	Yes	21	2	9.5	0.67
	No	229	29	12.7	

Detection of brucellosis in human, and its association with location, age, sex, and profession through RBPT

Higher occurrence was recorded in Tehsil Razzar (19.44%), followed by Lahore (16.95%), Swabi (3.51%), and Topi (0%) through RBPT. Location wise occurrence of brucellosis was found to be statistically significant (P<0.05). According to Age, occurrence was recorded higher in age group more than 45 years (14.28%), followed by age group less than 15 years (11.76%), age group 30-45 years (10.98%) and age group 15-30 years (9.24%). The occurrence of brucellosis through RBPT in different age groups was statistically non-significant (P>0.05). According to Gender, a higher occurrence of brucellosis was detected in female (18.18%) than, male (8.7%). The difference was close to the level of significance i.e. having P value (0.06) which shows that females are at higher risk for acquiring brucella infection as compared to males. Profession wise occurrence of brucellosis indicated that higher number

of cases were observed in housewives (21.05%), followed by field vets (12.24%), livestock farmers (10.86%), researchers (8.88%), farm workers (6.7%) and butchers (1%). Despite the numerical difference in the occurrence of brucellosis in different professions, it was found statistically non-significant (P>0.05) (Table 4).

Table 4. Detection of brucellosis in human in association with location, age, sex and professions through RBPT

Variables	Categories	Total sample (n=250)	Positive samples (n)	Positive percentage (%)	P-value
Location	Swabi	57	2	3.51	0.00*
	Topi	63	--	--	
	Lahore	59	10	16.95	
	Razzar	72	14	19.44	
Age	<15Y	17	2	11.76	0.95
	15-30Y	119	11	9.24	
	30-45Y	91	10	10.98	
	>45Y	24	3	14.28	
Sex	Female	44	8	18.18	0.06
	Male	207	18	8.70	
Profession	Field vets	49	6	12.24	0.6
	Farm workers	72	5	6.7	
	Livestock farmers	46	5	10.86	
	Butchers	20	2	1	
	Housewives	19	4	21.05	
	Researchers	45	4	8.88	

Detection of brucellosis in humans and its association with different factors through RBPT

The association between different factors and clinical history were also determined through RBPT. Those people who consumed raw milk (24%) were highly exposed to brucellosis as compared to those who were not (8.8%). The difference in the occurrence of brucellosis was significantly (P<0.05) associated with the consumption of raw milk. A statistically significant difference (P<0.05) was observed between the use of PPEs and not using PPEs. A high number of cases were observed in people not using PPEs (16.6%) as compared to those who were practicing PPEs (1.8%). Among clinical history, fever, joint pain and backache were significantly associated with the occurrence of brucellosis. Brucellosis was observed as 21.6, 19 and 33.3% of the human population having a fever, joint pain, and backache, respectively. However, the association of brucellosis was non-significant with other clinical signs i.e. body pain, night sweats and headaches.

The detection of brucellosis in humans and its association with different factors through RBPT is mentioned in the below (Table 5).

Table 5. Detection of brucellosis in humans and its association with different factors through RBPT

Variables	Categories	Total sample (n=250)	Positive samples (n)	Positive percentage (%)	P-value

Raw milk consumption	Yes	25	6	24	0.01*
	No	226	20	8.8	
PPEs	Yes	107	2	1.8	0.00*
	No	144	24	16.6	
Fever	Yes	60	13	21.6	0.00*
	No	191	13	6.8	
Joint pain	Yes	42	8	19	0.04*
	No	207	18	8.6	
Body pain	Yes	46	7	15.2	0.23
	No	205	19	9.2	
Night sweats	Yes	19	3	15.7	0.41
	No	232	23	9.9	
Backache	Yes	15	5	33.3	0.00*
	No	236	21	8.8	
Headache	Yes	21	1	4.7	0.40
	No	229	24	10.4	

Detection of brucellosis in human and its association with location, age, sex, and profession through i-ELISA

Indirect ELISA was conducted for the detection of both chronic (IgG) and acute (IgM) cases of brucellosis in humans and showed in the (Figure. 4 & 5). Samples screened from different tehsils of district Swabi revealed significant association ($P<0.05$) in the occurrence of brucellosis and location through IgM ELISA i.e. Lahore (27.1%), Razzar (12.5%), Swabi (5.3%) and Topi (1.6%). Through IgG ELISA, the occurrence of brucellosis was higher in Swabi (15.8%), followed by Razzar (15.3%), Lahore (13.6%) and Topi (3.2%) but the association was found non-significant ($P>0.05$). The occurrence of brucellosis with respect to age, sex and profession were found statistically non-significant ($P>0.05$) through both i-ELISA (Table 6).

Figure. 4. Positive control, negative control, cut-off (duplicate) and test samples results through Indirect ELISA (IgM) in human.

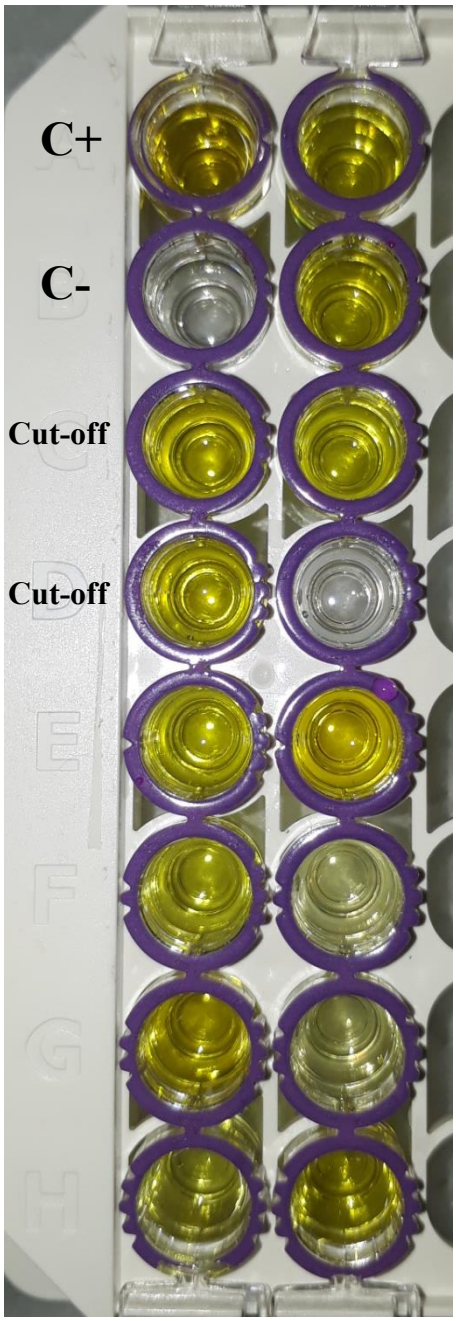

Figure. 5. Positive control, negative control, cut-off (duplicate) and test samples results through Indirect ELISA (IgG) in Human

Table 6. Detection of brucellosis in humans with respect to location, age, sex and professions through ELISA (IgM, IgG)

Variables	Categories	Total samples (n=250)	IgM ELISA			IgG ELISA		
			Positive samples (n)	Positive (%)	P-value	Positive samples (n)	Positive (%)	P-value
Location	Swabi	57	3	5.3	0.00*	9	15.8	0.09

	Topi	63	1	1.6		2	3.2	
	Lahore	59	16	27.1		8	13.6	
	Razzar	72	9	12.5		11	15.3	
Age	<15Y	17	1	5.9	0.42	--		0.32
	15-30Y	119	14	11.8		13	10.9	
	30-45Y	91	9	9.9		14	15.4	
	>45Y	24	5	20.9		3	12.5	
Sex	Female	44	7	15.9	0.32	6	13.6	0.7
	Male	207	22	10.6		24	11.6	
Professio-n	Field vets	49	8	17.4	0.6	9	18.4	0.38
	Farm workers	72	7	9.7		5	6.9	
	Livestock farmers	46	4	8.7		5	10.9	
	Butchers	20	4	20		2	10	
	Housewives	19	2	10.5		4	21.1	
	Researcher	45	4	8.9		5	11.1	

Detection of brucellosis in human through Indirect ELISA (IgM & IgG)

In high-risk human population were screened through indirect ELISA (IgM and IgG) and its association with different factors were analyzed statistically. The occurrence of brucellosis was detected 24% by both IgM and IgG ELISA in people who consumed raw milk of cattle as compared to those who were not (9.2 and 10.6% through IgM and IgG ELISA, respectively). The difference in the occurrence of brucellosis with respect to raw milk consumption was statistically significant ($P<0.05$). With respect to PPEs, brucellosis was recorded higher in those not practicing PPEs (18%) as compared to those adopted PPEs (2.8%) through IgM ELISA and the association was statistically significant ($P<0.05$). Similarly, through IgG ELISA, the number of cases was higher in people not using PPEs (15.2%) as compared to those using PPEs (7.4%). Though the association was not significant ($P>0.05$) but it was near to the level of significance i.e. having P value (0.06). The IgM ELISA detected a higher number of positive cases in a human having a fever (18.3%) as compared to those having no fever (9.4%). The difference was found to be non-significant ($P>0.05$), while close to the level of significance i.e. having P value (0.06). Likewise, the difference in occurrence of brucellosis through (IgG) ELISA was significantly higher in the human population having fever (20%) as compared to those having no febrile condition (9.4%). The detection of brucellosis through IgM and IgG ELISA, in high-risk human population with respect to another clinical history i.e. joint pain, body pain, night sweats and headache, was non-significantly associated ($P>0.05$). Higher occurrence of brucellosis through IgM ELISA was found significantly ($P<0.05$) recorded in people complaining backache (40%) as compared to those having no backache (9.7%). Instead of a higher number of brucellosis positivity cases in people suffering from backache (26.6%) as compared to those having no such complaint (11%), the difference was found to be non-significant ($P>0.05$), although it was near the level of significance (P 0.07) (Table 7).

Table 7. Detection of brucellosis in humans through Indirect ELISA (IgM & IgG)

Variables	Categories	Total sample (n=250)	IgM ELISA			IgG ELISA		
			Positive samples (n)	Positive (%)	P-value	Positive samples (n)	Positive (%)	P-value
Raw milk consumption	Yes	25	6	24	0.02*	6	24	0.05*
	No	226	21	9.2		24	10.6	
PPEs	Yes	107	3	2.8	0.00*	8	7.4	0.06
	No	144	26	18		22	15.2	
Fever	Yes	60	11	18.3	0.06	12	20	0.02*
	No	191	18	9.4		18	9.4	
Joint pain	Yes	42	6	14.2	0.55	6	14.2	0.62
	No	207	23	11.1		24	11.5	
Body pain	Yes	46	9	19.5	0.06	6	13	0.8
	No	205	20	9.7		24	11.7	
Night sweats	Yes	19	3	15.7	0.54	4	21	0.2
	No	232	26	11.2		26	11.2	
Backache	Yes	15	6	40	0.00*	4	26.6	0.07
	No	236	23	9.7		26	11	
Headache	Yes	21	1	4.7	0.3	2	9.5	0.71
	No	229	28	12.2		28	12.2	

Molecular detection in high-risk population and its association with location, age, sex, and profession of individual

Molecular detection in high-risk human population and its association with location, age, sex and profession of individual were analyzed. Brucellosis occurrences were found higher in Tehsil Lahore (13.6%), followed by Swabi (12.3%), Razzar (9.72%) and Topi (3.2%). The association with the occurrence of brucellosis to the location was non-significant i.e. (P>0.05). According to the age group, a few cases were higher in the age group 30-45 (12.1%), followed by age group 15-30 (8.4%), more than 45 years (8.3%) and less than 15 years of age (5.9%). Despite the numerical differences in different age groups, they found it not statistically significant. Similarly, sex-wise data was also non-significant i.e. P>0.05, with more number of cases recorded in females (13.63%) as compare to male (8.7%) and On the basis of professions, brucellosis were recorded higher in house-wives (21.05%), followed by field vets (16.3%), livestock farmers (10.9%), farm workers (6.9%), butchers (5%) and researchers (2.22%). According to professions, the difference was found non-significant i.e. P>0.05. The brucellosis in human with respect to location, age, sex and professions through PCR is showed in (Table 8).

Table 8. Brucellosis in human with respect to location, age, sex and professions through PCR

Variables	Categories	Total sample (n=250)	Positive samples (n)	Positive percentage (%)	P-value
Location	Swabi	57	7	12.3	0.2
	Topi	63	2	3.2	
	Lahore	59	8	13.6	

	Razzar	72	7	9.72	
Age	<15Y	17	1	5.9	0.76
	15-30Y	119	10	8.4	
	30-45Y	91	11	12.1	
	>45Y	24	2	8.3	
Sex	Female	44	6	13.63	0.31
	Male	207	18	8.7	
Profession	Field vets	49	8	16.3	0.09
	Farm workers	72	5	6.9	
	Livestock farmers	46	5	10.9	
	Butchers	20	1	5	
	Housewives	19	4	21.05	
	Researchers	45	1	2.22	

Molecular detection of brucellosis in humans and its association with different risk factors

Data for molecular detection of brucella antigen in high-risk humans and its association with different factors and clinical signs/history were given in (Table 9). Based on raw milk consumption history, the occurrence of brucellosis was recorded higher in individuals who consumed raw milk (28%) as compared to those who did not use raw milk (7.5%). The difference in the occurrence of brucellosis based on raw milk consumption was highly significant ($P<0.01$). According to the use of PPEs, number of cases were recorded in individuals not using PPEs (11.8%) as compared to those using PPEs (6.5%) but the difference was found non-significant ($P>0.05$). Similarly, a numerical difference was observed in other clinical signs/history, but the difference was recorded non-significant ($P>0.05$).

Table 9. Molecular detection of brucellosis in humans through PCR and its association with different factors

Variables	**Categories**	**Total sample (n=250)**	**Positive samples (n)**	**Positive percentage (%)**	**P-value**
Raw milk consumption	Yes	25	7	28	0.00
	No	226	17	7.5	
PPEs	Yes	107	7	6.5	0.16
	No	144	17	11.8	
Fever	Yes	60	9	15	0.1
	No	191	15	7.8	
Joint pain	Yes	42	3	7.1	0.54
	No	207	21	10.1	
Body pain	Yes	46	4	8.6	0.82
	No	205	20	9.7	
Night sweats	Yes	19	3	15.7	0.33
	No	232	21	9	
Backache	Yes	15	3	20	0.15
	No	236	21	8.8	
Headache	Yes	21	1	4.7	0.43
	No	229	23	10	

Discussion

Brucellosis is contagious and infectious zoonotic disease which causes infection in various mammalian species. In cattle, brucellosis results in reproductive losses such as abortion, retained placenta, still birth and sterility whereas in humans it causes the febrile condition, night sweats, backache, weakness, body pain and joints pain. Brucellosis is a well-known occupational disease of people who are involved in or deal with animals. Brucellosis is endemic in Pakistan and its prevalence has been reported from various parts of the country in livestock and human involved in the livestock industry. For successful limitation and eradication of brucellosis from animals and man, accurate diagnosis with base line surveillance data is of utmost importance. The current study was planned to examine, the occurrence of brucellosis in the high-risk human population in District Swabi of Khyber Pakhtunkhwa, Pakistan. Round about 250 blood samples (250 human) were collected and screened for brucellosis through SPAT, RBPT, ELISA and PCR. The overall detection of brucella immunoglobulin in tested cattle samples (n=250) was found to be 14, 7.6 and 8.8 %, through RBPT, i-ELISA and PCR, respectively. Parallel findings were reported, by Azmatullah et al. (2018) in Bannu, Khyber Pakhtunkhwa with a finding of 10%, and 11% positivity by RBPT and PCR, respectively. In another study conducted by (Hussain et al., 2008) in which the occurrence of disease was found to be 8% by ELISA and 10.2% by RBPT. The frequency of brucella infection in cattle was reported as, 14.70 and 18.53% through serology in government and private dairy farms by Nasir et al. (2004). Serological and Molecular detection of brucellosis, and its association within different breeds in sampled cattle i.e. Holstein Frisian (HF), Sahiwal, Jersey, non-descript, HF cross and Achai breed were detected as, 16, 20, 9.0, 21, and 0% respectively. This may be because exotic and cross-bred animals are more susceptible to various diseases as compared to local breeds. Further, these animals show more susceptibility due to their high productivity and less calving intervals which expose them to various pathogens including brucellosis. However, the high positivity in Sahiwal breed may be due to a lower number of tested animals which may be confirmed by investigating a large sample size of this breed. Which are in parallel to results of Ali et al. (2013) who investigated the occurrence of Brucella abortus in cattle and recorded highest sero-prevalence in crossed and imported cattle (7.8% and 6.0%, respectively). In another study carried out in Bangladesh, a high positivity of Brucella abortus was found in cross-bred cattle (6.28%) than in local breeds (0.85%) by (Sikder et al. (2012). However, Khan et al. (2016) investigation in Loralai, Baluchistan, reported higher occurrence of brucella infection in native cattle, breeds as compared to Holstein Frisian. On location basis detection of brucellosis in cattle was found to be statistically significant as previously reported in cattle by Ali et al. (2013) Pothohar Plateau, Pakistan. This might be due to the dependency of residents on agriculture crops production and livestock rearing for their livelihood, increase flow of trade animals and absence of quarantine practices in the study area.

The highest incidence rate of bovine brucellosis was found in > 7 years age group. This might be due to multiple calving and long exposure periods which are in parallel with findings of Khan et al. (2016). Significant association between age and infection was detected in cattle p- value (0.00), a similar association was reported by Aulak et al. (2008) and Mekonnen et al. (2010). In the present study positivity was detected at an elevated level in the cow (female), p- value (0.01) indicated significant association between sex of cattle and brucella infection. The increase incidenced in female was due to the normal production of erythritol during pregnancy which boosts production of Brucella abortus and faulty artificial insemination practices, as reported by (Gul and khan., 2007). Highest positivity was detected in animals with > 5 calvings. This might be due to multiple exposures to disease. The p values (0.00) indicate that there was a significant association between No. of calving and brucella infection. Matope et al. (2011) reported high prevalence was associated with maximum No. of calving. Sikdar et al. (2012) from Bangladesh reported high prevalence in animals having >4 calvings, which are in parallel to our findings. These results are in parallel with the findings of Deselegen and Gangwar (2011). The highest incidence

of brucellosis was detected in medium-sized farms (11-150 number of animals) i.e. 21% followed by household livestock (<10) i.e. 15% and 2% in large cattle farms (>150). This might be due to overcrowding and unsanitary hygienic measures. The p-value of (0.01) indicates that there exists significant association between herd size and brucellosis. The high brucellosis prevalence was reported in medium-sized farms (26-100 animals) than in small and large farms by Shome et al. (2014). Mohammed et al. (2011) in Nigeria reported higher prevalence rate in herd size (35-40 animals) as 4%. Their results demonstrated similar findings as an increase in the occurrence of brucellosis is directly proportional to greater herd size.

Higher incidence of brucellosis was recorded in animals with abortion history. The difference in the occurrence of brucellosis because of abortion history was significant i.e. $p < 0.05$. Ali et al. (2017) who reported abortion in the last trimester of pregnancy was associated with serological detection of brucellosis in cattle. In present study still birth and retained placenta were found to be statistically significant p-value (0.00) which agrees with Patel et al. (2015) who studied bovine brucellosis in reproductive disorders of dairy animals in peri-urban areas of Gujarat in the neighbor country. Status of animals and occurrence of brucellosis were recorded at the rate of: Pregnancy (6.6%), lactation (14.20%), and dry period (21.62%) which were found non- significant, p-value (>0.05). In a similar study conducted in Cameroon by Ndukum et al. (2018) reported a prevalence in pregnant cattle as 11 and 5.95% by RBPT and i-ELISA and found non-significant association in pregnancy and brucellosis sero-positivity. Higher detection of positivity was recorded in artificial insemination (A.I) group against natural mating animals. In a parallel study conducted by Shome et al. (2014) reported brucellosis as 20.17% in A.I and 5.38% in natural mating, which agrees with our findings. The higher brucellosis sero-positivity in study area may be due to use of semen from Brucella infected bull and through faulty insemination, use of same A.I gun for different animals.

Serological And Molecular Detection of Brucellosis in High-Risk Human Population

Detection of brucellosis was performed through SPAT, RBPT, Indirect ELISA (IgM, IgG) and PCR as 12.4, 10.4, 11.6, 12 and 9.6% respectively. A numerical difference was observed through detection of different tools, but the difference was statistically non-significant ($p>0.05$). Maria et al. (2019) detected 23% and 9% by RBPT and PCR respectively, in Swat, Kpk, Pakistan. Muhktar (2010) reported 21.7% of slaughterhouse workers through ELISA in Lahore. Farzana et al. (2015) carried out a study in District Charsadda KPK, reported an occurrence of 10 and 7.5% through SPAT and PCR respectively. Frequency of brucellosis in high-risk human population in Peshawar and Charsadda were reported at the rate 4.5, 9, 31, and 38% by RBPT, Competitive ELISA, FPA (Fluorescence Polarization Assay) and PCR by Mahmood et al. (2010). Ali et al. (2012) investigated prevalence of brucellosis in high-risk professionals in Potohar plateau, of Pakistan by using RBPT, SAT and RT-PCR detected as 6.9% by serology and 5.72 % by RT-PCR. Shahid et al. (2014) also recorded occurrence of brucella infection as 31.41% in 678 patients.Higher occurrence of brucellosis was recorded in Lahore and Razzar Tehsil as compared to Swabi and Topi Tehsils due to the dependency of residents on crop production and livestock rearing, and the last two tehsil are urban areas people life standard are advanced and educated. Positivity of brucellosis in different locations of study area was recorded as 7.02, 1.6, 20.4 and 19.7% in Swabi, Topi, Lahore and Razzar, respectively. Significance association was found between the occurrence of Brucellosis and locality. Ali et al. (2012) detected positivity in three different locations in study area as 11, 3.6 and 2.9% in Islamabad, Rawalpindi and Attock respectively. Likewise, variation in frequency of brucellosis in human from different localities was also found in a study conducted in Georgia (Havas et al. 2012). Higher occurrence was detected in the 30-45 years age group (14.3%). In a similar study, high brucellosis rate was detected in the 41-60 years age group as 12%. In another study conducted by Shahid et al. (2014) showed higher prevalence of (35.06%) in 41-60 age group. Further, in sex-wise investigation, high

positivity was detected in females, 15.9% while in males (10.6%). Shahid et al. (2014) also reported high incidence in female (37.06%).

Among high-risk human population i.e. field veterinarian, farm worker, livestock farmer, butchers, housewives and laboratory staff/ researcher were detected positive by SPAT showing a sero-positivity of 24.3, 8.4, 10.9%, 15, 21.1, and 2.2% in each group, respectively. A significant association was recorded in different professions of individuals and brucellosis occurrence in high-risk human population based on these four diagnostic techniques. In a similar study carried out by Shahid et al. (2014) also found prevalence of brucellosis to be 32.90, 32.67, 29.20 and 27.04%, in farmer, livestock owners, employees and other patients, respectively. Comparable results were reported by Ali et al. (2012) in category milkers (1.89%), livestock farmers (4.67%) and abattoir workers (16.7%). In another investigation, Farzana et al. (2015) also detected brucellosis at the rate of 11.42, 12.5, 0, 8.75 and 10.71% by SPAT while 8.57, 6.25, 0, 7.5, 7.14 and 3.75% by PCR in farmers, animal keepers, servants, housewives others. Results of all these studies showed that all mentioned professionals are high-risk groups for acquiring brucellosis. High positivity was recorded in individuals who consumed raw milk than in those individuals who did not. Consumption of raw milk was found to be a risk factor for contraction of brucellosis. Significant association was found between raw milk consumption and positivity for brucellosis. Similar results were reported by Ali et al. (2018) and Ndukum et al. (2018). Mukhtar (2010) also reported consumption of raw milk as risk for brucellosis infection in abattoir workers of District Lahore, Punjab, Pakistan.

Higher occurrence of brucellosis was detected in high-risk population who were not using Personal Protective Equipment's (PPE). The use of PPE has significant association with brucellosis infection (p value=0.00). Not using PPEs was found to be a risk factor for contraction of brucellosis in humans. Ndukum et al. (2018) also reported higher (14.13%) prevalence of brucellosis in individuals not using PPEs and 0% prevalence in those using PPEs.In individuals showing clinical symptoms of brucellosis i.e. fever, joint pain, body pain, night sweats, backache and headache, positivity was recorded as, 21.6, 16.6, 17.3, 21, 40 and 9.5%, respectively. Statistically, significant association of brucella positivity was found with febrile condition and backache (p value <0.05), while joint pain, body pain, night sweats and headache showed insignificant association. Memish et al. (2000) reported fever as 44% or fever with arthritis 42%. The prominent clinical symptoms observed were intermittent fever (71.62%) followed by joint pain and body aches (Shome et al. 2017).

Conclusion and Recommendations

Highest occurrence of brucellosis was detected in housewives mainly involve in domestic livestock rearing in rural areas. The animals related job/ profession of individuals was found to be a risk factor for contraction of brucellosis in human. Consumption of raw milk and non-existent personnel protective equipment's were found to be a risk factor for the contraction of brucellosis, furthermore pyrexia of unknown origin and backache were found to be significant. Awareness of high-risk population and public about brucellosis contraction and how to control it. Protective measures should be adopted by all personnel involved in animal-related operations, i.e. slaughtering, treatment, correction and assistance during reproductive problems. Consumption of raw milk should be avoided. Further epidemiological studies need to be conducted to explore the exact situation in humans on a national basis. Brucellosis should be dealt with under the umbrella of one health initiative to efficient control and eradication.

Conflict of Interest

The authors declare that there are not any potential conflicts of interest regarding the publication of this paper.

Data availability

The data used to support the findings of this study are included within the article.

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