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RESEARCH ARTICLE

Patho-Morphological Valuation of Acute Infection of *Brucella melitensis* in Goats

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ABSTRACT

Brucellosis caused by *Brucella melitensis* is an important zoonotic disease in the world. *Brucella melitensis* is the causative organism of caprine and ovine brucellosis characterized by abortion, retained placenta, orchitis which may induce infertility, epididymitis and rarely, arthritis in small ruminants. However, these lesions were regarded as chronic while acute patho-morphological lesions had not been described in goats earlier. This study investigates the histopathological lesions in organs of native goats of Pakistan acutely infected by *Brucella melitensis*. Grossly, granulomatous lesions and edema were observed in the lungs of the infected animals. Other organs including liver, spleen, heart and spleen showed no gross lesions during necropsy. Severe histological lesions were observed in the lungs and uterus of acutely infected goats. The organism was detected by PCR from liver, lungs, spleen, uterus, sub mandibular lymph nodes and bronchial lymph nodes. The organism was not detected by PCR from the kidneys, ovaries, mammary glands, supra mammary lymph nodes, internal ileac lymph nodes and scapular lymph nodes. It was concluded that *Br. melitensis* acute infection did not lead to clinical presentation, although the organism was well distributed in various organs of goats. The lesions were generalized, not limited to reproductive organs.

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INTRODUCTION

Brucella melitensis is Gram negative, facultative, intracellular, coccobacillus, highly pathogenic to a variety of animals and has zoonotic importance (Gul *et al*., 2015; Shahzad *et al*., 2017). *Brucella melitensis* is mainly widespread in Middle Eastern countries, Mediterranean and South America and Africa as well as in China, India and Central Asia (Anonymous, 2015). Goats and sheep are the natural hosts of *Br. melitensis,* although in a few European countries and central Asia it has emerged as an important problem in cattle (Zhang *et al*., 2015). Mainly it causes brucellosis with abortion in female goats and sheep, unilateral orchitis in case of males and intermittent fever in humans (Alton, 2015). Consumption of unpasteurized milk of infected goats and sheep and their products are the chief cause of infection in human (Wareth *et al*., 2014). *Br. melitensis* produces similar disease in goats as like *Br. abortus* that produces disease in cattle.

Animal generally abort only once, however, the organisms invade the uterus and shed in subsequent pregnancies through uterine fluids. Other clinical manifestations include: high frequency of still births, severe debilitation, disability, fever, anorexia, polyarthritis, pneumonia, abortion, reduced milk yield, retained placenta and prolonged calving interval with shedding of the pathogen in the milk and uterine discharges (Nielsen, 2002). The occasional occurrence of arthritis and hygroma are also reported (England *et al*., 2004). Death may take place as a consequence of retained fetal membranes or acute metritis (Gul *et al*., 2015).

Gross pathological lesions in brucellosis are primarily observed in the uterus, including hemorrhagic placentomes with multifocal fibrinous exudation. In advance cases, generalized lymphadenopathy, pleuritis and run-down appearance are also reported (Preman *et al*., 2013). Histologically lymph nodes showed hyperplasia and granulomatous lymphadenitis and placentomes with necrotic debris and several bacterial colonies surrounded

by an intense inflammatory infiltrate (Nasruddin *et al.*, 2014). Granulomatous inflammatory processes are also reported in mammary glands, liver, spleen, and kidneys. Central nervous system with multifocal or diffuse histiocytic meningitis, lymphoid depletion in thymus, lungs with diffusely thickened alveolar walls and interstitial inflammatory infiltrate are reported (Ahmed *et al.*, 2012).

The lesions in reported field cases were categorized as chronic while only one study describing acute lesions in the buck is available in the literature, however, no study describing acute lesions in goats is available. Moreover, studies to explore various lesions of brucellosis (*Br. melitensis*) in goats are also lacking. In view of the foregoing, the present study was planned to investigate the pathology in experimentally induced brucellosis in goats.

MATERIALS AND METHODS

Experimental animals and management: Eight goats were utilized for patho-morphological and molecular studies. Clinically healthy Barbri goats free from apparent ailment were utilized. Animals were kept in control house with 24±2°C temperature, 45-70% humidity and 12 hr light-dark cycle. Green fodder and water was available around the clock. All animals were tested for brucellosis by RBPT and ELISA prior to start of the experiment. Five animals were given infection of *Br. melitensis* through conjunctiva @ 0.5 mL/animal having bacterial concentration of 1 x 10^9/mL viable counts. Three animals were kept as control and inoculated with 0.5 mL of sterile normal saline. The animals were observed for clinical signs and symptoms of brucellosis like dynamics of rectal temperature twice daily, while total leukocyte counts (TLC) and presence of antibodies against *Brucella* through RBPT using the antigens procured from Veterinary Research Institute, Lahore, Pakistan and cELISA by using kit method (Savanova®, Sweden, Germany) were determined on alternative days. After three weeks of inoculation, animals were killed humanly. The morbid tissue samples including lymph nodes (mammary, internal iliac, bronchial), mammary gland, liver, spleen, lung and reproductive organs were collected and preserved in 10% buffered formalin.

Characterization through PCR: DNA was extracted from the above-mentioned tissues utilizing commercially available DNA extraction kit (Favorgen®, FABGK001) using the manufacturer's protocol. Then DNA was amplified using *Brucella* genus specific primers (B4- 5'-TGGCTCGGTTGCCAATATCAA-3' and B5- 5'-CGCGCTTGCCTTTCAGGTCTG-3') of *BCSP*-31 gene giving 223bp product for molecular detection of *Brucella* to check the localization of the organism (Baily *et al.*, 1992). PCR reactions were performed in a Thermal Cycler (BioRad®, T100™ Thermal Cycler) using standard protocol. Amplified product was run on gel electrophoresis and then visualized in gel documentation system (BioRad®, Gel Doc™ EZ Gel Documentation System).

Gross and histopathology: All organs were examined for gross lesions and morbid tissues were fixed in neutral buffered formalin. These tissues were processed by routine method of dehydration and paraffin embedding techniques. Sections of 3-4µm thick were cut and stained with hematoxylin and eosin. Slides were analyzed for histopathological lesions.

RESULTS

Clinical signs: In infected animals, fever was recorded 24 hrs PI which was persistent for two days in *Br. melitensis* infected animals. From day 4 onwards rectal temperature of infected goats became normal as compared with non-infected goats (Fig. 1A). A significant increase in the leukocytes was observed in infected animals at day 7 of the experiment which peeks at day 17 of infection and then started decreasing to its normal level as compared to non-infected goats (Fig. 1B). The infected animals were seropositive at day 9 post inoculation (PI) through RBPT (Fig. 1C) and cELISA (optical density) (Fig. 1D) and attained maximum titer at day 12 of the experiment which remained persistently high throughout the period of experiment. Animals kept as control showed no reaction to RBPT and cELISA (Fig. 1C and 1D).

Confirmation of the organism: By PCR, the organism (223 bp) was detected from the blood of the infected goats 24 hrs PI, then throughout the experiment (Fig. 2). At the end of the experiment, the organism (223 bp) was detected in the liver, lungs, spleen, uterus and sub-mandibular and bronchial lymph nodes. However, PCR could not detect organism from kidneys, ovaries, mammary glands and supra mammary, internal ileac and scapular lymph nodes (Fig. 3).

Gross and histopathology: Grossly, granulomatous lesions and pulmonary edema were observed in infected animals. Other organs including liver, heart and spleen did not show gross lesions during necropsy.

In histo-morphological examinations, lesions were observed in liver, lungs, kidneys, and uterus. There were hemorrhages around the vein, active von Kupffer cells along with mild mononuclear cells infiltration and coagulative necrosis in liver (Fig. 4a). There were multiple degenerating areas surrounded by infiltration of mononuclear cells. Hepatocytes appeared with centrally placed nuclei and granular cytoplasm with cloudy swelling, collapsed sinusoidal spaces and necrosis of hepatocytes (Fig. 4b).

Lungs showed diffusely thickened alveolar walls containing fibrinous exudate. Fibrinous exudate mixture of macrophages, mononuclear cells and granulocytes was present in alveoli and lungs parenchyma (Fig. 5). Lungs were also congested. Bronchioles contained fibrinous exudate (Fig. 5) and pleura were congested, thickened and even edematous and hemorrhages were seen just below pleura.

Kidneys showed infiltration of granular and multilobed cells in glomeruli obliterating the glomerular space with mild to moderate congestion of medullary area (Fig. 6a). Proximal as well as medullary tubules contained pinkish colored proteinaceous material. There were areas of necrosis and increased urinary spaces in glomerulus (Fig. 6b). Spleen showed hyperplasia of the many germinal follicles, proliferation of the cells with lightly stained cytoplasm and increased population of macrophages and red pulp was filled with lymphocytes, macrophages and plenty of RBC's.

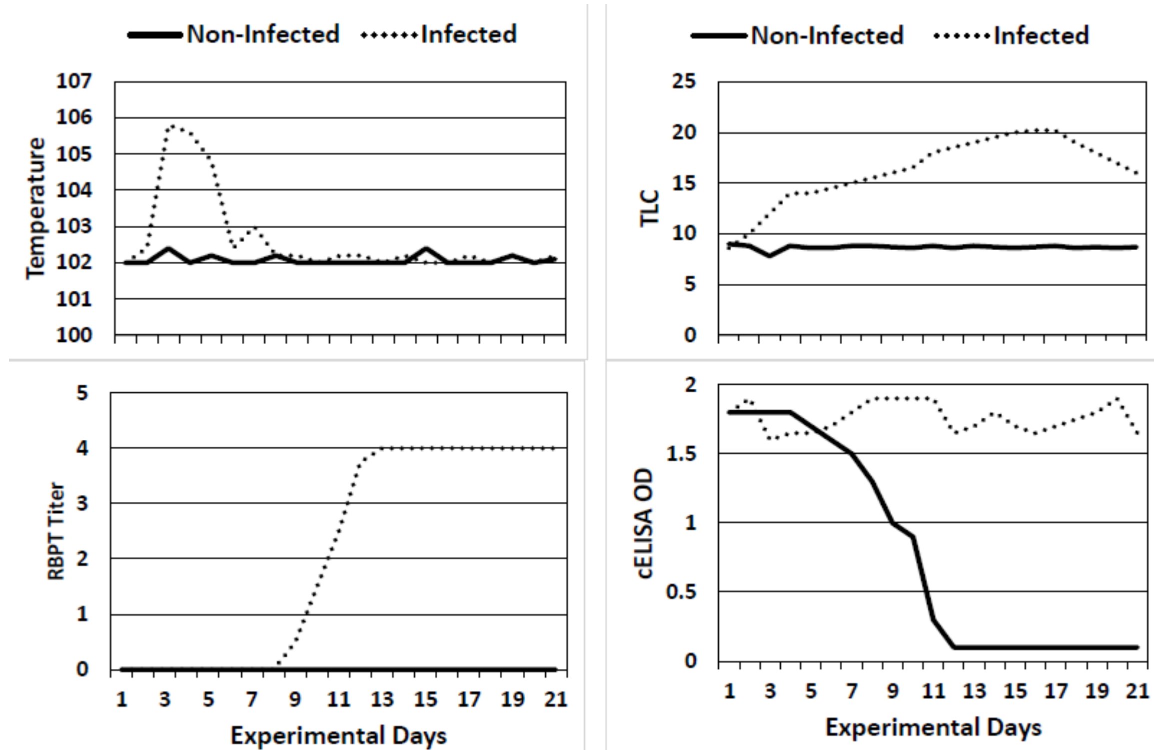

Fig. 1: Dynamics of goats (rectal temperature °F), TLC (10^3/µL), RBPT titer and cELISA (OD) infected with *Brucella melitensis* compared with non-infected goats at different time intervals.

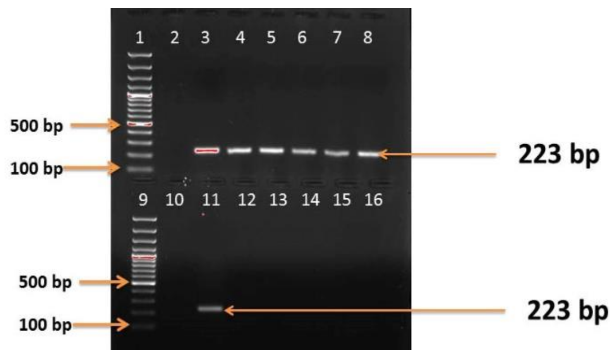

Fig. 2: Detection of *Brucella melitensis* from the blood of experimental goats. Lanes description: ladder (100bp): 1 & 9: Negative control; 2 & 10: Positive control; 3 & 11: Positive test samples; 4-8: Negative test samples; 12-14 and 15-16 wells were empty.

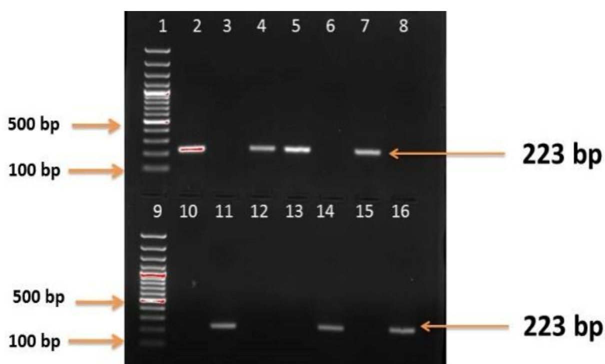

Fig. 3: Detection of *Brucella melitensis* from the different organs in experimental goats. Lanes description: Ladder (100bp): 1 and 9: Positive control; 2: Negative control; 3 & 10: Positive test samples; 4=Liver, 5=Lungs, 7=Spleen, 11=Uterus, 14=Sub-Mandibular Lymph Node (LN) And 16=Bronchial LN. Negative Test Samples: 6=Kidneys, 8=Ovary, 10=Mammary Glands, 12=Supra Mammary LN, 13=Internal Ileac LN and 15=Scapular LN.

Mammary gland showed focal interstitial infiltration of lymphocytes, macrophages, and neutrophils. Placentomes contained necrotic debris comprised of intense inflammatory infiltrate (Fig. 7A), dead tissue, multiple foci of degenerating areas specifically below the epithelium. Uterine glands were degenerated and mixed with inflammatory cells along with congested blood vessels (Fig. 7B).

DISCUSSION

Brucella melitensis was isolated from the spleen of a British soldier on the Island of Malta in 1887 by Sir David Bruce (Bruce) and was denominated *Micrococcus melitensis*. This bacterium was renamed as *Br. melitensis* in 1920, in honor of Dr. Bruce (Poester *et al.*, 2013). It causes significant problems in humans, goats and sheep around the world, however, pathogenesis is not yet clear. As goats are the primary hosts of *Br. melitensis*, using goats as model, in the present study, *Br. melitensis* was injected through conjunctiva to know the body dynamics and distribution of organism in various organs of goats.

In the present study, fever was observed in all animals infected with *Br. melitensis* (Fig. 1A) which agreed with the results reported earlier (Carvalho *et al.*, 2012). Contrary to our findings, no clinical signs were reported due to infection of *Br. melitensis* in bucks (Nasruddin *et al.*, 2014) and rams (Suraud *et al.*, 2008). The differences in results could be due to the variability in exposure time, route of infection and age of the animals.

Significantly increased leukocyte counts were recorded in the present study at day 7 of experiment which peaked at day 17 of infection (Fig. 1B). Increased leukocytes were also observed in bison from Yellowstone national park (Rhyan *et al.*, 2001). Antibodies against

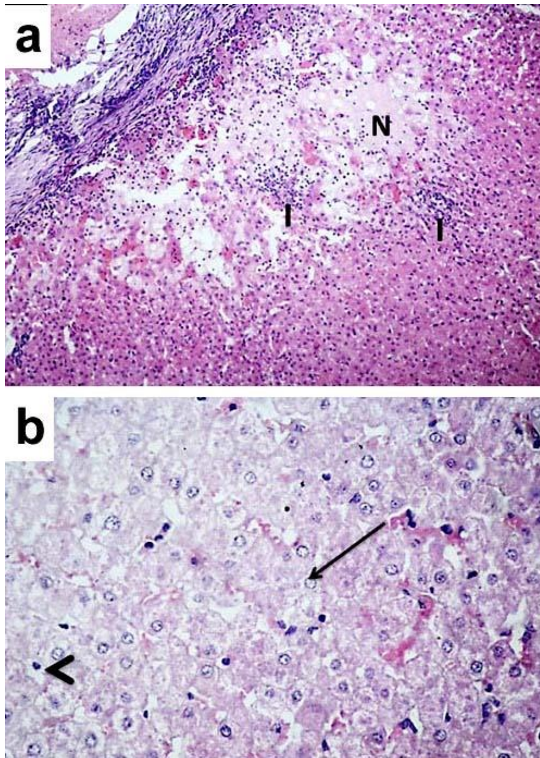

Fig. 4: Liver of goat infected with *Brucella melitensis* showing a) coagulative necrosis (N), hemorrhages and mild mononuclear cells infiltration (I), and b) cloudy swelling (arrow), collapsed sinusoidal spaces and individual cell necrosis of hepatocytes (arrow head). H & E stain. a=100X; b=200X.

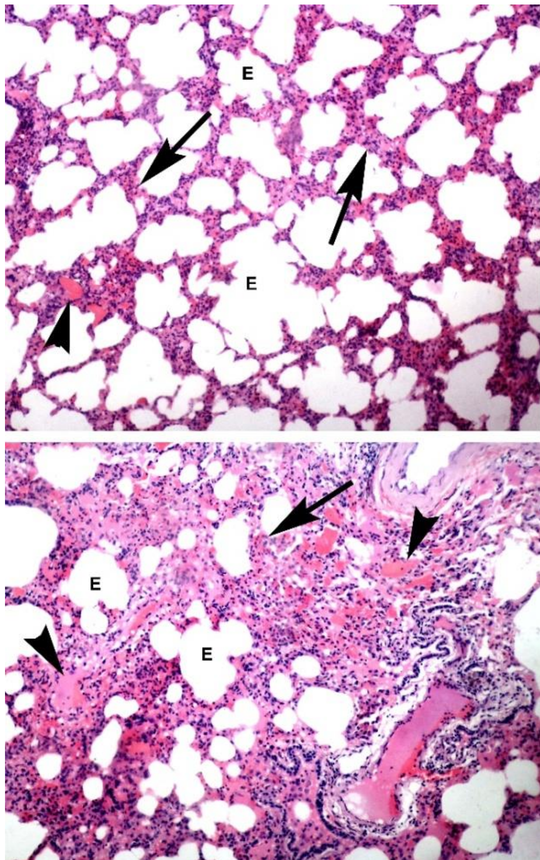

Fig. 5: Lungs of goat infected with *Brucella melitensis* showing emphysema (E), thickened alveolar walls (arrows) and alveoli filled with fibrinous exudate (arrow heads). Infiltration of inflammatory cells especially polymorphs around bronchiole is also evident. H & E Stain. 100 X.

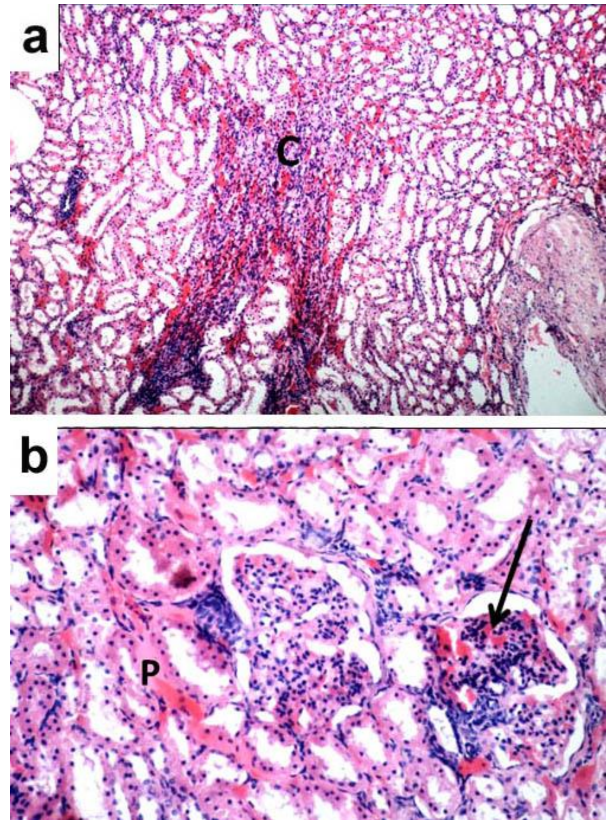

Fig. 6: Kidneys of goat infected with *Brucella melitensis* showing a) mild to moderate congestion (C) of medullary region and b) necrosis with infiltration of granular and multilobed cells in glomeruli (arrow), tubules with proteinaceous material (P) and increased urinary spaces in glomerulus. H & E stain. a=100X; b=200X.

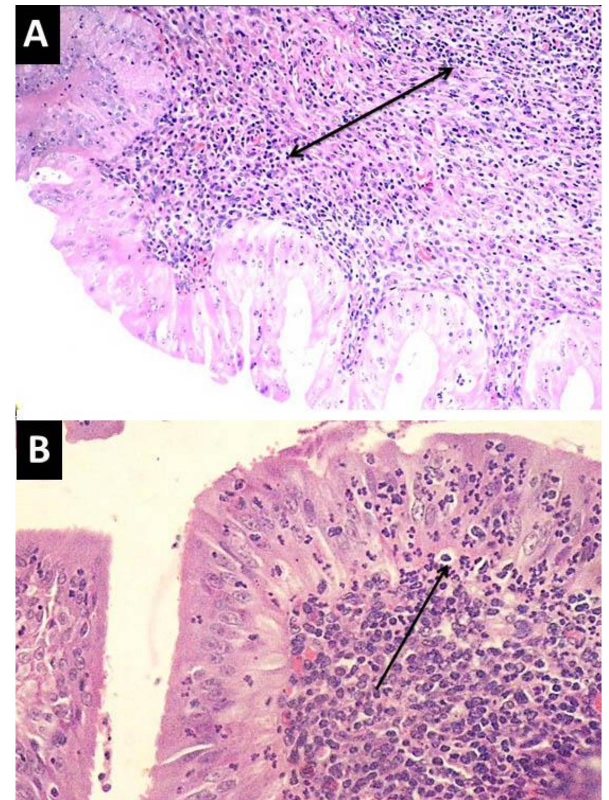

Fig. 7: Uterus of goat infected with *Brucella melitensis* showing A) necrotic debris comprised of intense inflammatory infiltrate (arrow), dead tissue and B) multiple foci of degenerating areas specifically below the epithelium. H & E stain. A=100; B=400X.

Brucella were detected at day 9 PI both by RBPT and cELISA which attain its maximum titer at day 12 of the experiment and remains constantly high throughout the experiment (Fig. 1C and 1D). Contrary to the present findings, antibodies against *Brucella ovis* were detected in experimentally induced infection in bighorn sheep at four weeks PI (McCollum *et al*., 2013). The difference in the results of both the studies could be the route of infection or sex (Nasruddin *et al*., 2014).

Through molecular investigations in the current study, the organism was successfully detected from the blood of the infected goats from day 2 PI to throughout the experiment. Moreover, PCR enabled us to understand how *Br. melitensis* disseminates in different tissues including liver, lungs, spleen, uterus, sub mandibular lymph nodes and bronchial lymph nodes (Fig. 3), moreover, the organism was not detected from kidneys, ovaries, mammary glands, supra mammary lymph nodes, internal ileac lymph nodes and scapular lymph nodes. No study in the published literature was available to compare the results of the present study. However, bacteriological examination in cattle infected with *Br. abortus* indicated widespread isolation of organism in different tissues including parotid, mandibular, suprascapular, retropharyngeal, supramammary, prefemoral, hepatic, popliteal, internal iliac, bronchial, renal lymph nodes and spleen (Forbes and Tessaro, 1996). *Brucella* was not isolated from the mesenteric lymph nodes, kidneys, liver, mammary glands and uterus (Xavier *et al*., 2009). It has been reported that the sensitivity and specificity values of *Brucella* obtained with PCR similar to that of bacteriology in semen, urine, preputial wash and tissue samples from infected rams (Xavier *et al*., 2010). Isolation and identification of *Brucella* is a low-yield process, prolonged turn over time and poses serious threat to laboratory personnel as well (Bhat and Asha, 2016). Serological tests are useful for screening large size herds, very easy to perform, convenient and cheap. Nevertheless, cross-reactions with non-specific antibodies may generate false positive results (Ahmed *et al*., 2016). Molecular diagnostic methods have considerably reduced this type of risk and are the most reliable tools in terms of sensitivity and specificity (Leyla *et al*., 2003). Moreover, agreement between PCR and bacteriology in study of Xavier *et al.* (2010) indicated that PCR method is highly efficient for the early diagnosis of brucellosis.

Histological findings of brucellosis in chronic cases includes abscesses, granulomatous lesions (caseating or non-caseating) and non-specific inflammation (Ahmed *et al*., 2012). Grossly, granulomatous lesions and edema were observed in lungs in the present study, these types of lesions have been reported in humans (Theegarten *et al*., 2008). Clinical signs and macroscopic lesions are contrary to those reported earlier by Nasruddin *et al.* (2014) in experimentally infected bucks with *Br. melitensis.* All other organs including liver, spleen, kidneys, testes and mammary glands were normal and no gross lesions were observed during necropsy probably due to the shorter exposure time and route of infection used.

While histo-pathological lesions observed in liver showed hepatocytes with centrally placed nuclei and granular cytoplasm with cloudy swelling give rise to collapsed sinusoidal spaces with individual cell necrosis of hepatocytes. Multiple areas of necrosis and hemorrhages around central vein with active von Kupffer cells and mild mononuclear cells infiltration were also evident. Nasruddin *et al*. (2014) also observed mild to moderate infiltration of inflammatory cells around central vein in the liver of experimentally infected bucks which shows hematogenous nature of distribution of the infection. Prior studies shown that *Brucella* can induce the release of pro inflammatory cytokines for instance interleukin IL-12 and tumor necrosis factor alpha for the influx of phagocytes. Afterwards the lesions progress into granuloma and expose the pathogens to phagocytosis (Zhan *et al*., 1996). Consequently, cytokine stimulatory properties of pathogen may describe the association stuck between tissue invasion and localized inflammation.

Lungs in the present study exhibited diffusely thickened alveolar walls, fibrinous exudate containing macrophages, mononuclear cells and granulocytes in alveolar spaces, interstitial inflammatory infiltrate in the parenchyma, congestion, dilatation of blood vessels, bronchioles containing fibrinous fluid, pleura thickened, edematous, congested and hemorrhagic just below pleura. Previous studies showed lungs with mild hyper-cellularity of interalveolar septae and infrequent macrophages, and randomly spread multinucleated cells in airways (Rhyan *et al*., 2001). Considerable involvement of lungs is an infrequent feature of brucellosis in Rhesus monkey (Mense *et al*., 2004). In humans, respiratory impediments are mostly mild flu-like with sore throat and mild dry cough. May cause pneumonia and lung abscess whereas serious manifestations are rare (Theegarten *et al*., 2008).

Kidneys showed infiltration of granular and multilobed cells in glomeruli obliterating the glomerular space with mild to moderate congestion of medullary area. Proximal as well as medullary tubules contained pinkish colored pretentious material and areas of necrosis were also evident at some places. Kidneys showed histologic indication of mild, multifocal nephritis characterized by lymphocytes infiltration predominantly in parivascular areas admixed with histiocytes and cellular debris (Mense *et al*., 2004). Histologically, kidneys are characterized by granulomas with central necrosis surrounded by multi-lobulated infiltrates with few giant cells in humans (Colmenero *et al*., 2002).

Spleen showed hyperplasia of the many germinal follicles, proliferation of the cells with lightly stained cytoplasm and increased population of macrophages, red pulp filled with lymphocytes, macrophages and plenty of RBC's. A study in rhesus macaques in experimentally induced by aerosol exposure of brucellosis also described increased numbers of lymphohistiocytic cells (Mense *et al*., 2004). In another study, lymphoid depletion was reported in spleen of naturally infected bovine which were sero-positive for brucellosis (Ahmed *et al*., 2012). These differences observed may be attributed to duration/course of the disease.

Mammary glands showed focal interstitial infiltration of lymphocytes, macrophages and neutrophils. Alton (2015) also described inflammation of the mammary gland in goats with acute natural infections. Uterus showed placentome with necrotic debris comprised of intense inflammatory infiltrate, dead tissue, multiple foci of degenerating areas specifically below the epithelium, glands are degenerated mixed with inflammatory cells and

blood vessels were congested. The results are in accordance with the results of Xavier *et al.* (2009) and Higgins *et al.* (2017) who recorded observations in *Br. abortus* infected cattle and goats. Lesions in uterus are characterized by granulomatous endometritis with infiltration of mononuclear inflammatory cells in uterine glands and lamina propria, along with fibrosis and calcification have been reported in naturally infected cow and buffalo (Ahmed *et al.*, 2012). Inflammatory response in uterus may provide a sign of localization of the organism. High amounts of erythritol have been suggested as a probable factor for assisting the localized progression of *Brucella* in uterine tissue (Poester *et al.*, 2013; Mai *et al.*, 2015). Thus, histopathological investigation of animals infected with *Brucella* may help in enlightening the distribution of infection and support in the diagnosis of brucellosis.

Conclusions: PCR used in this study validated to be highly useful for the diagnosis of *Brucella* at early stage of the infection. Present study also established that brucellosis produces numeral pathologic features, signifying that it may be valuable as a tool for evaluation of the vaccines. Beside this, conjunctival inoculation of *Brucella* provides its under-standings of immune-pathobiologic response in the host.

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Authors contribution: Research project was designed by AK, STG and MZK. Execution of the project was carried out by AS, MS, STG and MKS. AS wrote the manuscript while AK, DXX and JBY corrected and revised the manuscript. All authors approved the final version of the manuscript.

REFERENCES

Ahmed WA, Majeed SA, Abdul-Ameer AH, *et al.*, 2016. Sensitivity and specificity of various serological tests for detection of brucella spp. infection in male goats and sheep. Adv Microbiol 6:98-103.

Ahmed YFS, Sokkar SM, Desouky HM, *et al.*, 2012. Pathological studies on buffalo-cows naturally infected with *Brucella melitensis*. Global Vet 9:663-8.

Alton GG, 2015. *Brucella melitensis*. In: Animal Brucellosis. Nielsen K and JR Duncan (eds), CRC Press, Florida, USA; pp:379-82.

Anonymous, 2015. Office International des Epizooties, World Animal Health Information System (WAHID) Version 1. http://www.oie.int/wahis. Accessed on November 15, 2016.

Baily GG, Krahn JB, Drasar BS, *et al.*, 1992. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J Trop Med Hyg 95:271-5.

Bhat UP and Asha A, 2016. Brucellosis: An under diagnosed zoonosis. Int J Curr Microbiol App Sci 5:309-11.

Carvalho JCA, Moustacas VS, Xavier MN, *et al.*, 2012. Andrological, pathologic, morphometric and ultrasonographic findings in bucks experimentally infected with *Brucella ovis*. Small Rum Res 102:213-22.

Colmenero JD, Queipo-Ortuño MI and Maria Reguera J, 2002. Chronic hepatosplenic abscesses in brucellosis. Clinico-therapeutic features and molecular diagnostic approach. Diagn Microbiol Infect Dis 42:159-67.

England T, Kelly L, Jones RD, *et al.*, 2004. A simulation model of brucellosis spread in British cattle under several testing regimes. Prev Vet Med 63:63-73.

Forbes LB and Tessaro SV, 1996. Infection of cattle with *Brucella abortus* biovar 1 isolated from a bison in Wood Buffalo National Park. Can Vet J 37:415-9.

Gul ST, Khan A, Ahmad M, *et al.*, 2015. Epidemiology of brucellosis at different livestock farms in the Punjab, Pakistan. Pak Vet J 35:309-14.

Higgins JL, Gonzalezjuarrero M and Bowen RA, 2017. Evaluation of shedding, tissue burdens, and humoral immune response in goats after experimental challenge with the virulent *Brucella melitensis* strain 16M and the reduced virulence vaccine strain Rev. 1. PLoS One 12:e0185823.

Leyla G, Kadri G and Umran O, 2003. Comparison of polymerase chain reaction and bacteriological culture for the diagnosis of sheep brucellosis using aborted fetus samples. Vet Microbiol 93:53-61.

Mai HM, Irons PC and Thompson PN, 2015. Brucellosis, genital campylobacteriosis and other factors affecting calving rate of cattle in three states of Northern Nigeria. BMC Vet Res 11:7.

McCollum M, Rhyan J, Coburn S, *et al.*, 2013. Clinical, culture, serology, and histopathology outcomes of bighorn sheep experimentally infected with *Brucella ovis*. J Wildlife Dis 49:900-10.

Mense MG, Richard H, Wilhelmsen CL, *et al.*, 2004. Pathologic changes associated with brucellosis experimentally induced by aerosol exposure in rhesus macaques (*Macaca mulatta*). Am J Vet Res 65:644-52.

Nasruddin NS, Mazlan M, Saad MZ, *et al.*, 2014. Histopathology and immunohistochemistry assessments of acute experimental infection by *Brucella melitensis* in bucks. Open J Pathol 4:54-63.

Nielsen K, 2002. Diagnosis of brucellosis by serology. Vet Microbiol 90:447-59.

Poester FP, Samartino LE and Santos RL, 2013. Pathogenesis and pathobiology of brucellosis in livestock. Rev Sci Tech Off Int Epiz 32:105-15.

Preman P, Sanyal S and Das S, 2013. Histological changes in mammalian uterus in brucellosis. Asian J Biomed Pharm Sci 3:58-61.

Rhyan JC, Gidlewski T, Roffe TJ, *et al.*, 2001. Pathology of brucellosis in bison from Yellowstone National Park. J Wildlife Dis 37:101-9.

Shahzad A, Khan A, Khan MZ, *et al.*, 2017. Seroprevalence and molecular investigations of brucellosis in camel of selected regions of Pakistan. Thai J Vet Med 47:207-15.

Suraud V, Jacques I, Olivier M, *et al.*, 2008. Acute infection by conjunctival route with *Brucella melitensis* induces IgGþ cells and IFN-g producing cells in peripheral and mucosal lymph nodes in sheep. Microbes Infect 10:1370-8.

Theegarten D, Albrecht S, Tötsch M, *et al.*, 2008. Brucellosis of the lung: case report and review of the literature. Virchows Arch 452:97-101.

Wareth G, Melzer F, Elschner MC, *et al.*, 2014. Detection of *Brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR. J Infect Dev Countries 15:1339-43.

Xavier MN, Paixao TA, Poester FP, *et al.*, 2009. Pathological, immune-histochemical and bacteriological study of tissues and milk of cows and fetuses experimentally infected with *Brucella abortus*. J Comp Pathol 140:149-57.

Xavier MN, Silva TM, Costa EA, *et al.*, 2010. Development and evaluation of a species-specific PCR assay for the detection of *Brucella ovis* infection in rams. Vet Microbiol 145:158-64.

Zhan Y, Liu Z and Cheers C, 1996. Tumour necrosis factor alpha and interleukin 12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. Infec Immun 64:2782-6.

Zhang J, Yin S, Guo F, *et al.*, 2015. Activation of the PI3K/Akt pathway is essential for the survival of *Brucella melitensis* 16M in vitro and in vivo. Pak Vet J 35:7-12.