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Full Length Article

Identification of 23 kD Immunogen from Native Antigens of *Babesia bigemina* in Splenectomized Calf

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Abstract

Primers were designed to amplify 18S RNA sequences of *Babesia bigemina* and *B. bovis*. Blood samples were collected from naturally infected calves. PCR products of sizes 321 bp and 269 bp, were obtained for *B. bigemina* and *B. bovis*, respectively. Infected RBCs (iRBCs) were then, cultured in *in vitro* to achieve >10% parasitemia of *B. bigemina*. Splenectomized calf was infected with 1×10^8 iRBCs. Three intact calves aged 4 to 6 months were infected with iRBCs with *B. bigmenia* from splenectomized calf. Then, merozoites of *B. bigemina* were harvested from iRBCs of splenectomized calf and were analyzed through immuno-blotting through homologous and heterologous sera collected from field and experimentally infected calves. SDS-PAGE was performed to analyze the protein bands of native antigens of *B. bovis* and *B. bigemina*. The gel was transferred on to nitrocellulose membrane containing native antigens of *B. bigemina*. The membrane was immuno-blotted with serum sample from *B. bigemina*-positive animal and it showed a clear band of about 23 kD, while this band was absent on membranes which were incubated with sera from *B. bovis*-infected calf and negative control animal. Further, native antigens of *B. bigemina* were coated for optimization of indirect ELISA with homologous serum of experimentally infected intact calves. Cut off point ≥ 0.4893 was considered positive. We obtained antibody titer of 149 ± 97.8 to 299 ± 196 in wells of microtiter plate coated with 5 μg/mL of native antigens while this titer was from 597 ± 391 to 1195 ± 782 in wells coated with 10 μg/mL of antigens. This putative protein would need to be characterized in future to validate its diagnostic value for screening field samples. © 2020 Friends Science Publishers

Keywords: Bovine babesiosis; *Babesia bigemina*; Indirect ELISA; Western Blot; Merozoite Antigens; SDS-PAGE

Introduction

Babesia spp. are apicomplexan protozoan parasites, which are the cause of bovine babesiosis (McCosker 1981), predominantly in exotic cattle in Pakistan (Ali *et al.* 2016). Globally, babesiosis in cattle is caused by four species of *Babesia*; *B. bigemina*, *B. bovis*, *B. divergens* and *B. orientalis* (Uilenberg 2006; Ica *et al.* 2007; Altay *et al.* 2008). *Babesia bigemina* and *B. bovis* transmitted by *Rhipicephalus microplus* are prevalent principally with significant economic impact in livestock industry in Pakistan (Durrani and Kamal 2008; Nieto *et al.* 2012; Zulfiqar *et al.* 2012; Hassan *et al.* 2018; Rehman *et al.* 2019). *Babesia divergens* has not been reported in Pakistan (Jabbar *et al.* 2015), while *B. orientalis* has been reported recently in scarce in some parts of Pakistan (Rehman *et al*. 2019). Microscopy is not reliable tool for the diagnosis of *Babesia bigemina* in cattle (Salih *et al.* 2015) because of misdiagnosis for different *Babesia* spp. causing babesiosis in cattle (Böse *et al.* 1995).

DNA based diagnosis like PCR are gaining popularity for the detection of parasites nowadays (Wong *et al.* 2014). PCR is more specific and sensitive than microscopic diagnosis of *B. bigemina* and *B. bovis* in cattle (Oliveira-Sequeira *et al.* 2005). *Babesia* infections are difficult to detect because of the low number of parasites in peripheral blood. PCR had been developed to detect *Babesia spp.* with great advantages, such as high analytical sensitivity and specificity rates (Criado-Fornelio 2007). Different PCR formats have been used, one of the first PCR assays performed was that developed for detection of *B. bigemina* (Figueroa *et al.* 1992). PCR can detect *Babesia* in acute or chronic form of babesiosis in cattle even with its small number (Calder *et al.* 1996).

Western blot is an immunoassay which is primarily used for identifying the immunogens specific for blood parasites like *Theileria annulata* (Bilgic *et al.* 2016), *Leishmania donovani* (Singh and Sundar 2017),

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Trypanosoma cruzi (Bucio *et al.* 1999), *Babesia divergens* (Gabrielli *et al.* 2012), *Babesia microti* (Ooka *et al.* 2011) and *Babesia bigemina* (Posnett *et al.* 1998). The antigens extracted from erythrocytes infected with *Babesia* could be used for the evaluation of immunogens in *B. bovis* (Mahoney *et al.* 1981) and *B. bigemina* (Goldman *et al.* 1972) infections in cattle. Firstly, we screened the erythrocytes infected with *B. bigemina* from the field samples through PCR and then immunogen equivalent to 23 kD was found specific for *B. bigemina* through Western blot assay. Lastly, ELISA was performed by coating native antigens to evaluate the antibody titer in experimental *B. bigemina*-infected calves.

Materials and Methods

Source of parasite

Babesia bigemina local strain was obtained from the blood sample of the calf (designated as K) at the exotic cattle farm located sub-urban of Lahore (31.4330° N, 74.1945° E), Pakistan. Aseptically, a total of 20 mL of blood was collected in 50 mL syringe containing Ethylenediamine Tetra Acetic acid (EDTA) having 18-gauge needle. In order to keep number of infected erythrocytes (iRBCs) the parasite was maintained in MASP (MicroAerophilic Stationary Phase) culture system as described (Levy and Ristic 1980) in cell culture laboratory at the department of Parasitology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. To increase the parasitemia, the iRBCs were inoculated in splenectomized calf (designated as KS) (Mahoney *et al.* 1973) which was the source of infection for the experimental calves (4 to 6 months of age) designated as A1, A2 and A3. The calves were infected according to the protocol described elsewhere (Figueroa *et al*. 1992). Briefly, 1×10^8 iRBCs were inoculated in calf (Ramírez *et al.* 2011) through intravenous route and the infection was later confirmed through Polymerase Chain Reaction (PCR). The collection and inoculation of *B. bigemina,* is outlined in Fig. 3.

Primer design and PCR

A pair of oligonucleotides was designed for targeting the 18S ribosomal RNA gene. The sequences of different isolates of *B. bigemina* and *B. bovis* were retrieved from Genbank and were aligned initially by BioEdit software (Hall 1999) and then consensus sequence was created. Then, the primers were designed by using Geneious R8 software (Talundzic *et al.* 2015). Genomic DNA was extracted from 200 μL of blood samples through DNA extraction kit (GeneAll®, Exgene™, 105-101) according to the manufacturer instructions, while quantity of DNA was measured through NanoDrop spectrophotometer (Thermo Scientific 2000/2001, Wilmington, DE 19810, USA). PCR was used for confirmation of *B. bigemina* and *B. bovis* by using specific primer pairs: Bg-forward: AGAGGGACTCCTGTGCTTCA, Bg-reverse: GACGAATCGGAAAAGCCACG and Bv-forward: AATATGGGTTGGGCAATGCG, Bv-reverse CCACCCAAAACAAGAGCAACT, respectively. PCR was performed according to the protocol described elsewhere (Mtshali and Mtshali 2013; Farooqi *et al.* 2017) with little modifications. Briefly, PCR reaction was carried out in 20 μL of reaction mixture containing 1 μL of each primer pair (10 pmol), 2 μL of DNA, 10 μL of 2X AmpMaster™ Taq (GeneAll®, Exgene™, 541-001) and 6 μL of UltraPure™ DEPC water (Cat no. 750023; Invitrogen, Carlsbad, CA, USA). The control samples were run for each reaction. The annealing temperature was 60°C for *B. bigemina* and 56°C for *B. bovis* with 35 cycles each for the PCR reaction. The amplified DNA was subjected for electrophoresis in 1.5% agarose gel (120 V, 200 mA, 45 min) stained with ethidium bromide (Cat no. 15585-011; Invitrogen, Carlsbad, CA, USA) and observed under GelDoc 100 imaging system. DNA ladder of 100 bp (Genedirex, Catlogue # DM001-R500) was used to compare the amplified product of PCR.

Preparation of native antigens

Merozoites of *B. bigemina* were harvested from iRBCs according to the protocol described elsewhere (Ruiz *et al.* 2001) with modifications as follows. Twenty milliliters of whole blood were centrifuged for plasma and erythrocyte separation at 1000 × *g* (BIOShield Swing-out Bucket Rotor, Catalogue # 75003182, Thermo Fischer,) at 4°C for 6 min. Supernatant was discarded and the pellet of cells was washed thrice with 1X Phosphate Buffer Saline (PBS) following centrifugation at 1000 × *g*. Immediately after centrifugation, the pellet was treated with 3 parts of cold ammonium chloride lysis buffer (0.17 *M*) for one minute (Podoba and Stevenson 1991). Reaction was stopped by adding RPMI-1640. The mixture was centrifuged at 1000 × *g* for 15 min and the erythrocyte-free pellet was washed three times in PBS. The pellet was resuspended in 5 volumes of PBS containing protease inhibitor (1 m*M* PMSF, 2 m*M* TPCK and 0.1 m*M* TLCK). *B. bigemina* merozoites were disrupted by repeated freeze/thaw method in liquid nitrogen. The supernatant obtained after centrifugation at 10,000 × *g* for 1 h at 4°C was stored at -20°C. Quantification of the Ag was assessed through BCA Kit (Bicinchoninic Acid) (Cat. 786-570, G-Biosciences®), following manufacturer's protocol. Antigens were prepared for SDS-PAGE, Western Blot analysis and Enzyme Linked Immuno-Sorbent Assay (ELISA). Antigens from splenectomized calves were prepared after each month for 12 months post-infection.

Collection of sera

The sera were collected from the animals (A1, A2, A3, K and N). The calves (A1, A2 and A3) were infected experimentally with iRBCs containing *B. bigemina* from the

splenectomized calf while K and N samples were obtained from natural *B. bigemina*-infected calf at acute stage of the disease and *Babesia*-free calf, respectively. Absence or presence of infection was confirmed through PCR. N was kept as negative control, which was collected from the calf of tick-free area. Blood of N was screened negative for any parasite through microscopy and PCR. The sera were collected from experimentally infected calves (A1, A2, and A3) in tubes after 15 days and 30 days post-infecton without anticoagulant and were stored at -20°C. The sera were collected from splenectomized calf every month until 12 months post-infection.

SDS-PAGE and immunoblotting

Native antigens were analyzed through Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot according to the protocol described elsewhere (Nabi *et al.* 2017). In Brief, 10 μg of native antigens were loaded on each lane, separated by (12% w/v) polyacrylamide gel and transferred to nitrocellulose membranes (NCM, 0.22 μm, Trans-Blot® Turbo™ Midi nitrocellulose transfer packs#1704159, Bio-Rad, USA) using Trans-Blot® Turbo™ transfer system (Bio-Rad, USA). Blots were cut into strips, labeled and blocking was achieved with skimmed milk (5% w/v) in TBS buffer (Tris-buffered saline 20 m*M* Tris-HCl, pH 7.2, 150 m*M* NaCl). Sera collected from infected animals were used as a source of primary antibody. Following washing, the strips were then charged with goat anti-bovine IgG-alkaline phosphatase conjugate (1:10,000) secondary antibodies (ThermoFisher USA, Catalogue # A18754. NBT/BCIP (bioWORLD, USA) was used as chromogenic substrate.

Indirect ELISA

ELISA was performed according to the protocol described elsewhere (Ruiz *et al.* 2001; Naeem *et al.* 2018). Briefly, 5 μg/mL or 10 μg/mL of native antigens were coated in 96 well ELISA plate (BIOFIL®, Guangzhou, China) in 50 m*M* bicarbonate buffer, incubated at 4ºC overnight. The ELISA plates were washed 3 times with washing buffer (0.05% Tween 20, 0.01 *M* PBS, pH 7.2). Saturation of the microtiter plate was done with 4% BSA in PBS followed by incubation at 37ºC for 2 h. Both negative and positive sera were diluted in PBS to achieve two-fold serial dilutions. Diluted sera were poured into each well. The plate was incubated again at 37ºC for 1 h. Second washing was performed with washing buffer as described previously. Bound antibodies were detected by incubating at 37ºC for 2 h with goat anti-bovine IgG-alkaline phosphatase conjugate (1:10,000). After washing thrice, phosphatase activity was measured with P-nitrophenyl phosphate (pNPP, Cat. 41480004-1, Bioworld® USA) at 1 mg/mL in 1 *M* diethanolamine (Cat. 40400060-3, Bioworld®). Optical density (OD) values were obtained by ELISA reader (Elisa reader, Model ELx 800, BioT, USA) at wavelength of 405 nm.

Results

PCR

Field blood samples of *B. bigemina*-infected, *B. bovis*-infected and experimental *B. bigemina*-infected animals were confirmed through PCR. Product sizes; 321 bp and 269 bp, were obtained along with control positive DNA for *B. bigemina* and *B. bovis*, respectively as shown in Fig. 1. The PCR products were also confirmed through sequencing.

Identification of *B. bigemina*-specific immunogen

Infected or non-infected RBCs were analyzed through SDS-PAGE. Several bands of various sizes were revealed through Coomassie Blue staining (Fig. 2A). SDS-PAGE was also done with crude antigens harvested from iRBCs of splenectomized calf at 12 months post-infection. The gel then, was transferred to the nitrocellulose membrane and it was immuno-blotted with *B. bigemina*-positive, *B. bovis*-infected and negative sera. The blots revealed a specific band of about 23 kD when they were incubated with *B. bigemina*-positive serum sample. The blots incubated with negative and *B. bovis* sera, did not reveal any specific band at 23 kD (Fig. 2B).

Analysis of IgG antibodies through Indirect ELISA

Immune response in *B. bigemina*-infected intact calves were evaluated against 5 μg/mL and 10 μg/mL concentrations of native antigens through indirect ELISA. The antibody titer in experimentally infected calves with *B. bigemina* was from 149 ± 97.8 to 299 ± 196 in wells coated with 5 μg/mL while this titer was from 597 ± 391 to 1195 ± 782 in wells coated with 10 μg/mL of native antigens. The antibody titer was observed in 3 calves at 15 days and 30 days post-infection. This titer was not significant between the two concentrations.

Discussion

We screened the erythrocytes infected with *B. bigemina* from the field samples through PCR. Splenectomized calf was infected with iRBCs from naturally infected calf and then 23 kD immunogen specific for *B. bigemina* was identified through Western blot assay in *B. bigemina*-infected splenectomized calf at its carrier state. Finally, ELISA was performed with native antigens to find out the antibody titer in *B. bigemina*-infected intact calves.

DNA based diagnosis like PCR are gaining popularity for the detection of parasites nowadays (Wong *et al.* 2014). PCR is more specific and sensitive than microscopic diagnosis of *B. bigemina* and *B. bovis* in cattle (Oliveira-Sequeira *et al.* 2005). PCR can detect *Babesia* in acute or

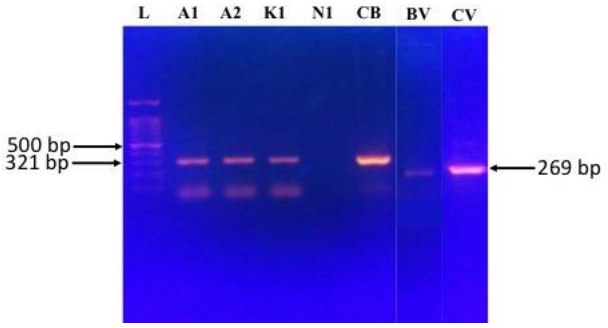

Fig. 1: PCR results of blood samples for *Babesia bigemina* and *Babesia bovis*. L is 100 bp ladder. A1 and A2 are experimentally infected animals. K1 and BV are field samples positive for *B. bigemina* and *B. bovis* respectively. CB and CV are control positive DNA of *B. bigemina* and *B. bovis* respectively. 321 bp and 269 bp are PCR products of *B. bigemina* and *B. bovis* respectively. N1 is the control negative DNA. PCR was performed at least n=3 to confirm the results

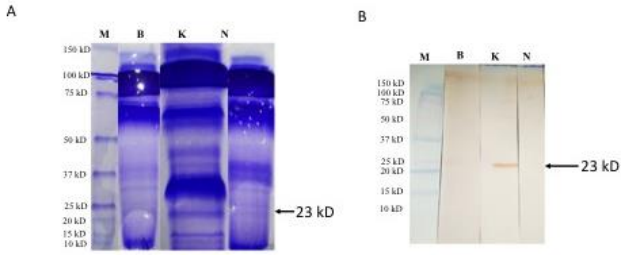

Fig. 2: Vertical gel electrophoresis and Western-blot analyses in non-reducing conditions. (**A**) SDS-PAGE analysis stained with Coomassie blue. M is protein marker. B and K are total lysate of *B. bovis* and *B. bigemina* respectively. N is the total lysate of control negative sample. (**B**) Western-blot results. M is protein marker. B and K are positive serum samples of *B. bovis* and *B. bigemina* respectively. N is the negative serum sample. About 23 kD protein band was revealed with serum sample of *B. bigemina* while B and N did not reveal any specific band when the blot was transferred from gel run with total lysate of *B. bigemina.* SDS-PAGE and Western Blot analyses were performed at least n=3 to confirm the results

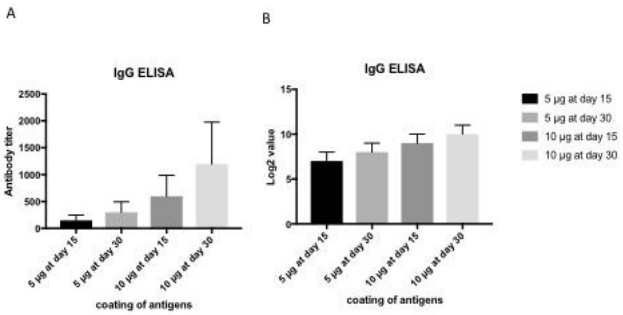

Fig. 3: Evaluation of IgG antibodies through ELISA by coating 5 and 10 μg/mL of antigens. Serum samples of all experimental *B. bigemina*-infected animals were tested with 2-fold serial dilutions. (**A**) graph plotted with Antibody titer, and (**B**) graph plotted with Log2 values. ELISA was performed at least n=3 to get an average of the results

chronic form of babesiosis in cattle even with its small number (Calder *et al.* 1996). We have detected *Babesia bigemina* and *Babesia bovis* infections from the infected cattle through PCR. We achieved 321 bp and 269 bp PCR products by using specific primers for *B. bigemina* and *B. bovis*, respectively. Nested or semi-nested PCR is used for the differential diagnosis of *B. bigemina* and *B. bovis* on non-quantitative thermal cycler (Herrera *et al.* 2017; Sivakumar *et al.* 2018). Oliveira-Sequeira *et al.* (2005) used two sets of primer pairs (external and internal primer pairs) for two round PCRs. They achieved 278 bp and 350 bp PCR products for the detection of *B. bigemina* and *B. bovis* respectively by using external primers. Our primers were more specific because we did only single round PCR. Nested PCR is needed when external primer pair is less specific (Szöllősi *et al.* 2008). Dissimilar to our results, Durrani and Kamal (2008) obtained 1124 bp and 541 bp product sizes through molecular detection of *B. bigemina* and *B. bovis*, respectively (Durrani and Kamal 2008). The difference in results is due to the use of different primer pairs.

Merozoite is the invasive stage in the life cycle of *Babesia*, which has surface antigens (Madruga *et al.* 1996) serve as receptors to get attachment with ligands on the RBCs (Yokoyama *et al.* 2002). Immunoassays like western blot technique can be used for the identification of immunogens of different *Babesia* spp. like *B. divergens* (Gabrielli *et al.* 2012), *B. microti* (Ooka *et al.* 2011), and *B. bigemina* (Posnett *et al.* 1998). The antigens extracted from erythrocytes infected with the *Babesia* could be used for the evaluation of immunogens in *B. bovis* (Mahoney *et al.* 1981) and *B. bigemina* (Goldman *et al.* 1972) infections in cattle. We have identified an immunogen about the size of 23 kD from *B. bigemina* isolate propagated in splenectomized calf by immuno-blotting its native antigens with the sera of *B. bigemina* and *B. bovis* -infected cattle. Our splenectomized calf became carrier of *B. bigmenia*. Previously, splenectomized calf had been used for the propagation of *Babesia* spp. (Callow *et al.* 1979; Mahoney *et al.* 1981; Figueroa *et al.* 1992; Posnett *et al.* 1998). We obtained only single band of protein at carrier state of splenectomized calf as compared to the acute state of the babesiosis caused by *B. bigemina*, while in acute state we obtained numerous bands through western blot analysis (Data not shown). Kahl *et al.* (1982) have demonstrated the variation of protein expressions between virulent and avirulent strains of *B. bovis* through 2-D gel electrophoresis. Montenegro-James *et al.* (1987) obtained immunogens of 23, 26 and 29 kD from merozoites of *B. bigemina* strain isolated from Venezuela through western-blot analysis by incubating with homologous serum sample (Montenegro-James *et al.* 1987). Posnett *et al.* (1998) transferred the native antigens from Kenyan strain of *B. bigemina* to the nitrocellulose membrane from SDS-PAGE gel and immuno-blotted with sera of *B. bigemina* Kenyan strain-infected, *B. bigemina* Mexican strain-infected and *B. bovis*-

infected cattle. They found about 50 kD immunogen specific for Kenyan strain of *B. bigemina* only, which was not recognized by the sera of both Mexican strain of *B. bigemina* and *B. bovis* (Posnett *et al*. 1998). Environmental factors, genetic variation and host adaptability of pathogen may determine the phenotype of the parasite (Kaltz and Shykoff 1998; Gandon and Michalakis 2002). Moreover, splenectomized calf was used for propagation of vaccine strains to get high parasitemia and it may become carrier or premuned (Hussein 1977).

Immuo-assays like IFAT and ELISA have been used employing native or crude antigens (Böse *et al*. 1995; Pipano *et al.* 2002; Shkap *et al.* 2007) to evaluate vaccine response against babesiosis caused by *B. bigemina*. ELISA has been used for the detection of *B. bigemina* (Molloy *et al.* 1998; Battsetseg *et al.* 2018; Jaramillo *et al.* 2018; Sivakumar *et al*. 2018; Obregon *et al.* 2019), which is more cost-effective than IFAT (Böse *et al*. 1995). There are several cross-reactive proteins between *B. bigemina* and *B. bovis* (Figueroa *et al.* 2006) so specific protein would be feasible to be used for development of ELISA against *B. bigemina* in naturally infected animals. Our *B. bigemina*-specific 23 kD protein could be used for specific ELISA in both acute and carrier states (Löhr 1972; Goo *et al.* 2009) either in native or recombinant protein form as a source of antigen after characterization.

We used ELISA coated with native antigens of *B. bigemina* for the evaluation of humoral immune response of experimentally infected intact calves. ELISA with native antigens is not considered fit to evaluate immune response in naturally infected animals because there are similarities of antigens between *B. bigemina* and *B. bovis* (Morzaria *et al.* 1992). Native antigens of *B. bigemina* had been used to detect antibodies (Goldman *et al*. 1972) by using IFAT (Bessenger and Schoeman 1983) and ELISA (El-Ghaysh *et al.* 1996). Bessenger and Schoeman (1983) has obtained antibody titer of 170 ± 114.9 and 480 ± 184.8 at 3rd and 4th week respectively, in post *B. bigemina*-infected animals by using IFAT (Bessenger and Schoeman 1983). E1-Ghaysh *et al*. (1996) coated the ELISA plates with 12 μg/mL native antigens with 1/10,000 dilution of conjugate. They evaluated 2 positive sera of *B. bigemina* 2–4 week-infected cattle and obtained the optical density (OD) value of 1.425 ± 0.4596 (El-Ghaysh *et al*. 1996). We obtained antibody titer of 149 ± 97.8 to 299 ± 196 (OD value: 1.657 ± 0.1066) in wells coated with 5 μg/mL of native antigens while this titer was from 597 ± 391 to 1195 ± 782 (1.717 ± 0.2071) in wells coated with 10 μg/mL of antigens. The variation in results is due to the difference of *B. bigemina* strains and quantity of antigen coated.

Conclusion

Immunogenic protein of about the size of 23 kD was found specific for our local *B. bigemina* strain in splenectomized calf at its carrier state 12 months post-infection. ELISA was performed with native antigens to find out the antibody titer in *B. bigemina*-infected intact calves. This immunogen specific to *B. bigemina* will be characterized and its diagnostic value will be determined in future.

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Ethical Approval

The study was approved from the animal welfare and ethic society of University of Veterinary and Animal Sciences Lahore, Pakistan with No. DR 1112, Dated: 13-10-2017. All the animals were treated after experiments.

Author Contributions

Conceptualization, Muhammad Imran Rashid, Haroon Akbar, Aneela Zameer Durrani; methodology, Muhammad Imran Rashid, Haroon Akbar; formal analysis, Muhammad Imran Rashid, Haroon Akbar; investigation, Umber Rauf, Shafqat Shabir, Matiullah Khan, Imran Rashid, Haroon Akbar; resources, Muhammad Imran Rashid, Haroon Akbar; data curation, Umber Rauf, Shafqat Shabir, Matiullah Khan writing—original draft preparation, Muhammad Imran Rashid, Umber Rauf, Shafqat Shabir, Matiullah Khan; writing—review and editing, Muhammad Imran Rashid, Umber Rauf, Shafqat Shabir, Matiullah Khan, Haroon Akbar, Aneela Zameer Durrani; visualization, Muhammad Imran Rashid, Haroon Akbar, Umber Rauf, Shafqat Shabir, Aneela Zameer Durrani; project administration, Muhammad Imran Rashid, Haroon Akbar; funding acquisition, Muhammad Imran Rashid, Haroon Akbar

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