Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.054

RESEARCH ARTICLE

Study on Tissue Tropism and Molecular Detection of VP2 Gene of Infectious Bursal Disease Virus in Experimentally Infected Broiler Tissues

Beenish Zahid*[1], Asim Aslam[1], Rukhsana Anjum[2], Saeed Imran[1], Irfan Irshad[1], Chaman Ara[3] and Haleema Sadia[4]

[1]Department of Pathology, University of Veterinary and Animal Sciences, Lahore, Pakistan; [2]Poultry Research Institute, Rawalpindi; [3]Department of Zoology, University of the Punjab, Pakistan; [4]Center for Applied Molecular Biology (CAMB), University of the Punjab, Lahore
*Corresponding author: bz-leo@hotmail.com

ARTICLE HISTORY (18-008)

Received: January 09, 2018
Revised: April 22, 2018
Accepted: April 23, 2018
Published online: June 26, 2018

Key words:
Immunohistochemistry (IHC)
Infectious bursal disease viruses
Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
Specific Antigen Negative (SAN) Chicks

ABSTRACT

The Infectious bursal disease virus (IBDV), rapidly destroy immature B lymphocytes in bursa of fabricous and cause the immunosuppression and high mortality in broiler chicken. Therefore, present study was conducted to investigate the viral antigen in lymphoid tissues of experimentally infected broiler chicken and specific antigen negative (SAN) chicks through immunohistochemistry (IHC) and reverse transcriptase polymerase chain reaction (RT-PCR). For this purpose, two hundred broiler chicks were reared and divided into 4 groups (A, B, C and D), fifty birds in each group. The birds in group A were commercial broiler chicks having maternal derived antibodies and group B containing SAN chicks. The groups A and B were challenged with IBDV field isolate. While the group C and D was unchallenged control. On 3rd, 5th, 9th and 14th day post infection (pi), Lymphoid organs Bursa of Fabricius, spleen and thymus and non lymphoid organs kidney and liver were collected for histopathology, IHC and viral genome detection by RT-PCR. At day 3 of pi, viral antigen observed in lymphoid cells of follicles of bursa and thymus, marked leukocytic infiltration occurred with bursal oedema and hyperemia. Virus was constantly found at 5th day post infection until day 14th, in the primary lymphoid tissues and characterized in acute inflammation. The hypervariable region of viral antigen, VP 2 gene was detected in bursa, thymus, spleen, kidney and liver tissues in challenged groups A and B through RT-PCR by using specific primers of 743bp. The virus antigen detection from tissues reduced with increasing antibody titer. It is concluded, the IBDV antigen detection rate in primary lymphoid organs was high as virus targets the lumphocytes and macrophages. Immunohistochemistry and RT-PCR are more specific and sensitive methods for detection of IBD viral antigen.

To Cite This Article: Zahid B, Aslam A, Anjum R, Imran S, Irshad I, Ara C and Sadia H, 2018. Study on tissue tropism and molecular detection of VP2 gene of infectious bursal disease virus in experimentally infected broiler tissues. Pak Vet J, 38(3): 291-295. http://dx.doi.org/10.29261/pakvetj/2018.054

INTRODUCTION

Infectious bursal disease (IBD) also called Gumboro disease is a significant threat to flourishing industry being highly spreading infection. The disease is caused by Birna virus, a double stranded RNA virus having two segments (Razia *et al*., 2014). It is associated with high mortality in young chicken 3 to 6 week of age. The infectious bursal disease virus (IBDV) causes lymphoid depletion of the bursa of Fabricius that results in significant depression of humoral antibody response and leads to immunosuppression.

Histopathological lesions in bursa of Fibrinous were observed; lesions included changes in cytoplasm organelles, nuclear chromatin margination, degeneration and necrotic lymphocytes and macrophages (Sing *et al*., 2015). The lymphoctic depletion and damage at cortex and medulla of immune follicles observed at one day of post IBDV infection. The depleted and damaged lymphocytic cells were replaced by reticuloendothelial cells, heterophils, necrotic debris and mononuclear cells. All lymphoid follicles are affected by 3 or 4 days post infection and the increase in weight of BF seen at this time is caused by severe oedema, hyperaemia and marked accumulation of heterophils (Lukert and Saif, 2004). The cyst developed at medullar region of lymphoid pellicles and on bursal epithelium at the end of immunological

reaction. The heterophils and plasma cells occur and fibroplasiais observed in intrafollicular connective tissues in inflammation. The tissue bursal samples were preserved in 10% formalin as fixative purpose to maintain the normal morphology of cell, in order to detect the IBDV antigen in tissue immunohistochemical technique used and Universal Vector stain Kit was used for immunohistochemical staining. The rabbit anti IBD virus antibodies were raised in rabbit to detect the virus in targeted tissues (Nafi *et al*., 2017).

The IBDV viral strains characterization and to determine the genetic diversity of IBDV was done by DNA sequencing. Mostly protocols of reverse transcriptase- polymerase chain reaction and procedures are based on the viral protein (VP2) nucleotide sequences. Now Quantitative real time polymerase chain reaction new procedures published based on (VP1) (Raue and Mazaheri, 2003). To detect the initial stage of IBD infection new PCR named In Situ RT-PCR developed to determine the infection in bursa of Fabricius (Zhang *et al*., 2002). In present study Reverse-Transcriptase-Polymerase-Chain Reaction were applied to detect the pathogenic virulent strain of IBDV by designing new primers that hit the hypervaribale region of Vp2 nucleotide of IBDV. An Immunohistochemistry and RT-PCR technique helps to identify the IBD viral antigen in lymphoid tissues samples.

MATERIALS AND METHODS

Isolation and Identification of Infectious Bursal Disease Virus (IBDV): A total of 100 samples of Bursa of Fabricius were collected from broiler poultry forms of district Punjab for virus isolation. Bursa of Fabricius were triturated using phosphate buffered saline (PBS) and 0.1ml of triturate was injected in 16 embryonated chickens eggs (9 day of age) via chorio-allointoic membrane (CAM) route while 5 embryos were taken as control. Candling was performed on daily basis and any mortality up to 48 hrs was discarded, however; dead embryos after 48 hrs were used for further processing. Embryos along with CAM were collected, triturated and again inoculated in 9 day old chicken embryonated eggs (2^{nd} passage) (Tesfaheywet *et al*., 2012). The IBDV was identified through agar gel precipitation test by the method of Okwor *et al.* (2011) and Reverse transcriptase polymerase chain reaction (Beenish *et al*., 2017). Embryo infectious dose 50 (EID 50) of isolated field IBDV estimated according to the method described by Reed and Muench (1938).

Experimental design: Two hundred day old broiler chicks were procured and reared under standard housing conditions in University of Veterinary and Animal Sciences, Lahore, the birds were fed with commercially prepared feed. The humoral antibody titer against IBD of reared birds were checked through Enzyme linked immunosorbent assay in order to check the status of maternally derived antibodies both in commercial broilers and Specific Antigen Negative (SAN) chicken at 0 day. The SAN chicks were serologically negative for IBD. The birds were divided into 4 groups (A, B, C and D), fifty birds in each group. The birds in group A were commercial broiler chicks having maternal derived antibodies and Group B SAN chicks were challenged with IBDV field isolate at the rate of 0.1ml of EID50 (virus titer $10^{5.50}$/100µl) on 2^{nd} week of age through eye drops (Abdel-Alim and Saif, 2001). While the group C and D was unchallenged control containing commercial and specific antigen negative chicks.

Histopathological examination: On day 3^{rd}, 5^{th}, 9^{th} and 14^{th} of post infection, from each group five birds were slaughtered, gross and histopathological lesions on lymphoid organs and non lymphoid organs were observed (Subtain *et al*., 2011).

Immunohistochemistry (IHC) of Collected Tissues: Paraffin-embedded tissue sections were mounted on positively charged super frost plus glass slides. *Invitrogen Super Picture™ 3rd Gen IHC Detection Kit Cat. No. 87893* was used for immunohistochemistry of tissues according to the manufacturer instruction procedure.

Producing hyper immune sera: To raise hyperimmune sera 10 healthy adult rabbits (male) were taken from the market around the area of Lahore. The serum samples were collected and checked by Enzyme linked immunosorbent assay (ELISA) for antibodies against IBDV and found zero. The inoculums was prepared with IBDV virus with an adjuvant, injected subcutaneously (s/c) @ 0.2 ml per rabbit and bled periodically to check the antibody titer in their serum against IBDV through ELISA (Ahmed *et al*., 2015).

Reverse transcriptase polymerase chain reaction (RT-PCR): TriZol reagent was used for viral RNA extraction from tissue samples (Bursa, thymus, spleen, liver, kidney and lung). cDNA was synthesized using eluted RNA with transcriptor first strand cDNA synthesis Kit® (Fermantas, USA) according to manufacturer's instructions. Extracted DNA amplified using specific VP2 gene primers set **F**: GCCCAGAGTCTACACCAT **R**: CCCGGATTATGTCT TTGA of 743bp (Roussn *et al*., 2012; Sara *et al*., 2014).

RESULTS

Clinico-pathological characteristic: The clinical sign of infectious bursal disease virus was assessed in commercial broiler chicks having maternal derived antibodies (MDA) and specific antigen negative (SAN) birds, as compared with control. The broiler chickens were apparently normal, no clinical signs were observed in first twenty four hours of post infection in all treatment groups. Birds of group B showed clinical signs 72hrs after infection with signs of listlessness, weakness and loss of appetite. All IBDV-inoculated birds presented severe prostration, listlessness and whitish diarrhea (Table 1); birds died or were slaughtered on 3^{rd}, 5^{th}, 9^{th} and 14^{th} day of post challenge. In group A clinical signs like ruffled feathers and dullness were noticed on day 5^{th} of infection. At the end of day 5 post-infection, three and one birds died in groups B and A respectively. All chickens challenged to infectious bursal disease virus were dull, depressed, feed and water consumption was decreased and had ruffled feathers at 9^{th} days post infection (dpi) (Table 1).

Table 1: Scoring of clinical signs 3rd, 5th, 9th and 14th days post infection in experimental chicken infected with IBD virus strain

Groups	Days post infection	Listlessness	Ruffled feathers	Whitish diarrhea
A	3 day	+	+	+
	5 day	++	++	++
	9 day	+++	+++	+++
	14 day	++	++	+
B	3 day	++	++	++
	5 day	+++	+++	+++
	9 day	+++	+++	+++
	14 day	++	++	++
C	3 day	_	_	_
	5 day	_	_	_
	9 day	_	_	_
	14 day	_	_	_
D	3 day	_	_	_
	5 day	_	_	_
	9 day	_	_	_
	14 day	_	_	_

(-) = absent; (+) = mild; (++) = moderate; (+++) = severe Group A: Commercial chicks having maternal derived antibodies IBDV infected chicks, Group B: Specific antigen negative IBDV infected chicks, Group C: Commercial chicks control, Group D: SAN control.

Table 2: Histopathological changes at 9th day of age post infection

Groups	Lymphoid Organs			Non-Lymphoid Organs		
	Bursa	Thymus	Spleen	Kidney	Liver	Lung
A	++	++	+	+	+	-
B	+++	+++	+	++	+	-
C	_	_	_	_	_	_
D	_	_	_	_	_	_

(-)= absent; (+) = mild; (++)= moderate; (+++)= severe Group A: Commercial IBDV infected chicks, Group B: Specific antigen negative IBDV infected chicks, Group C: Commercial chicks control, Group D: SAN control.

Table 3: Histopathological changes at 14th day of age post infection

Groups	Lymphoid Organs			Non-Lymphoid Organs		
	Bursa	Thymus	Spleen	Kidney	Liver	Lung
A	+	+	+	+	+	-
B	++	++	+	+	+	-
C	_	_	_	_	_	_
D	_	_	_	_	_	_

(-)= absent; (+) = mild; (++)= moderate; (+++)= severe Group A: Commercial IBDV infected chicks, Group B: Specific antigen negative IBDV infected chicks, Group C: Commercial Chicks control, Group D: SAN control.

Table: 4: IBD virus antigen detection in different organs through reverse transcriptase polymerase chain reaction

Groups	Total No. of samples	Tissues	Positive on day 3rd	Positive on day 5th	Positive on day 9th
A	6	Bursa	1	2	2
	6	Thymus	1	1	1
	6	Spleen	1	1	1
	6	Liver	0	0	0
	6	Kidney	1	1	0
	6	Lung	0	0	0
B	6	Bursa	2	2	2
	6	Thymus	1	2	1
	6	Spleen	2	2	1
	6	Liver	1	0	0
	6	Kidney	1	2	1
	6	Lung	0	0	0
C	6	Bursa	0	0	0
	6	Thymus	0	0	0
	6	Spleen	0	0	0
	6	Liver	0	0	0
	6	Kidney	0	0	0
	6	Lung	0	0	0
D	6	Bursa	0	0	0
	6	Thymus	0	0	0
	6	Spleen	0	0	0
	6	Liver	0	0	0
	6	Kidney	0	0	0
	6	Lung	0	0	0
Total	144				

Group A: Commercial IBDV infected chicks, Group B: Specific antigen negative IBDV infected chicks, Group C: Commercial chicks control, Group D: SAN control.

Gross and histopathological changes: Five birds from each group were slaughtered on 3rd, 5th, 9th and 14th days of post infection of IBDV. Gross and histopathological lesions in lymphoid and non lymphoid organs scored on different days. On 3rd day of postinfection histologically bursal changes observed in group A were minimal, in group B mild changes were observed in lymphoid organs. On 5th day postinfection moderate histological changes were observed in different organs in group A, severe changes were observed in group B. Typical histological changes associated with IBDV infection, including lymphoid depletion, necrosis and hemorrhages in bursa (Fig. 1A & B). Bursal changes include follicular necrosis, necrosis of eptithelial cells, cyst formation and marked fibroplasias (Fig. 1C). Severe hemorrhages and lymphocytic depletion at medullar region of thymus were observed in group B (Table 2) on day nine of post infection. Moderate changes were observed on 14th day in challenged group (Table 3). Thymus showed moderate to severe changes include congestion, hemorrhages, lymphocytic necrosis at medullar region and thickening of interlobular connective tissues in challenged groups. Mild changes were observed in liver of group B include hepatocyte necrosis and congestion (Fig. 1D). Mild lymphocytic infiltration was observed in kidney of group B. Group C and D control groups showed no specific lesions.

Immunohistochemistry: Immunohistochemical staining for virulent field strain of IBDV described rapid spread of the viral antigen in infected tissues and spreading rate was maximum in 3rd to 14th day of post infection. The intensity of positive immunohistochemistry staining was higher in 3 to 9 dpi and viral antigen positive cells observed in the infected lymphocytes and macrophages. Antigen observed in the cytoplasm of cells. The IBDV antigen positive cells were observed through immunohistochemistry in infected lymphocytes and macrophages at medullary area of thymus and bursal lymphoid follicles (Fig. 2A and B). Virus was constantly found at 3rd day post infection until day 14th, in the primary lymphoid tissues like thymus and bursa of Fabricius and characterized in acute inflammation. At day 3, viral antigen was observed in lymphoid cells of follicles of thymus, marked leukocytic infiltration with bursal oedema and hyperemia (Fig. 2 F). On day 5th post infection, antigen was detected through immunohistochemistry in medullar and cortical region of follicles with marked lymphoid depletion and virus replication resulted in changes like lymphoid necrosis in bursal follicles and marked fibrous tissue proliferation observed (Fig. 2D). On 9th day of post infection in specific antigen negative chicken proliferation of epithelial layer of bursa produced glandular structure consisted columnar epithelium that contains mucin globules (Fig. 2C). On 14th day group B containing specific antigen negative birds, bursal folds regression observed, and viral antigen infected cells spreaded in surroundings. The IBDV antigen positive cells were observed in epithelium of bursa.

IBD virus antigen was detected in lymphoid organs of bursa, thymus and spleen and non-lymphoid organs of kidney, liver and lung on 3, 5 and 9 day post infection through reverse transcriptase polymerase chain reaction (Table 4).

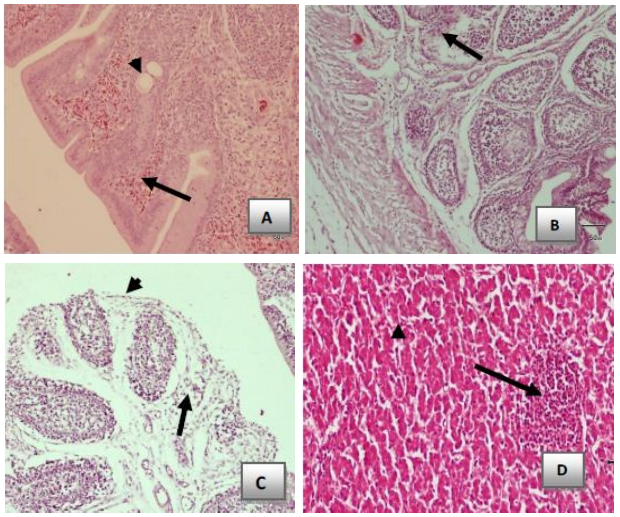

Fig. 1: **A**; Bursa from group B 5th day of post challenged showed hemorrhages (arrow) and follicular cyst formation (arrowhead). **B**: Bursa from group A 5th day of post challenged showed severe lymphocytic depletion, infolding of epithelium and cyst formation. **C**: H&E staining of Bursa from group B at 14th day of PI severe lymphoid follicular atrophy (arrow) and degraded epithelial cells (arrowhead). **D**: H&E staining of Liver from group B at 5th day of PI. Marked leukocytic infiltration (arrow) observed in hepatocytes. (H& E Stain 100X)

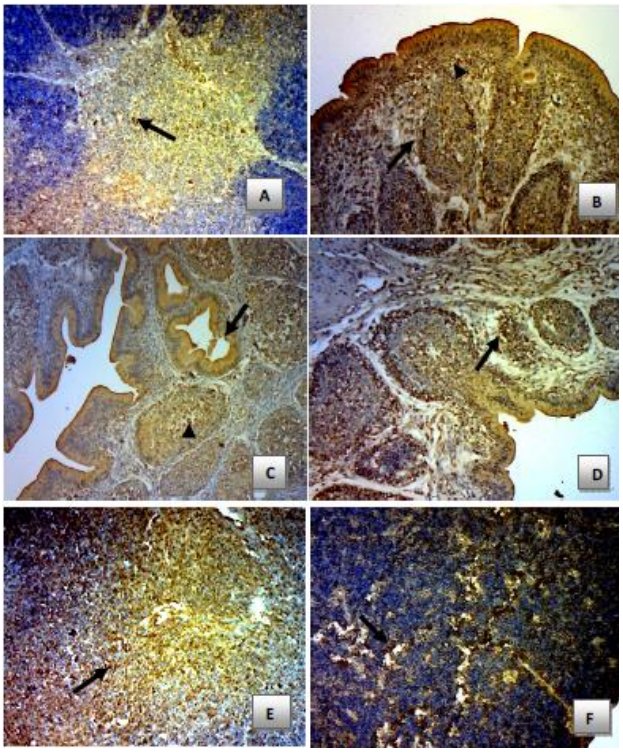

Fig. 2: A: IHC staining of Thymus from group A at 5th day PI. Antigen mainly observed in the medullar region of lymphoid follicle. **B:** IHC staining of Bursa of Fabricius from group B at 5th day PI. Brownish viral antigen mainly observed in the cortex (arrow) and medulla of lymphoid follicle and edematous sub-epithelial area (arrowhead). **C:** IHC staining of Bursa of Fabricius from group B at 9th day PI. Antigen observed in degenerated lymphoid cells of medullar and cortical region (arrowhead) with glandular structure (arrow) formation. **D:** IHC staining of bursa of Fabricius from group B at 9th day PI. Viral antigen observed in the degenerated lymphoid cells in lymphoid follicles in diffuse pattern with fibroplasia. **E:** IHC staining of Spleen from group A at 5th day PI. Antigen observed in degenerated lymphoid cells around central artery. **F:** IHC staining of Thymus from group B at 3rd day of PI. Antigen mainly observed in the thymus lymphoid follicles at central region (IHC Staining 100X).

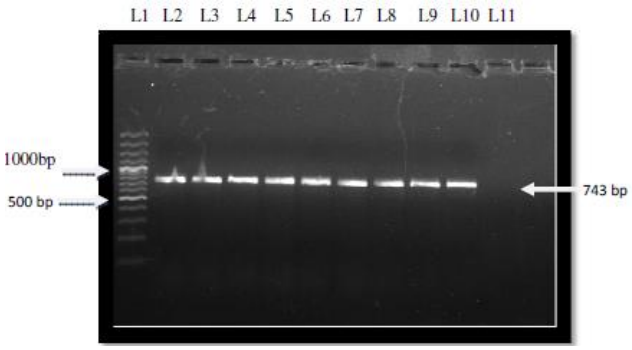

Fig. 3: Virus detection in tissue samples of IBD infected birds by RT-PCR. L1=DNA ladder 100bp, L2-L3=Thymus Positive tissues Samples, L4-L6=Bursa positive tissue samples, L7-L8=Spleen Positive tissue samples L9-L10= Kidney positive tissue samples L11: Negative sample.

Reverse transcriptase polymerase chain reaction: VP2 nucleotide was detected by using specific primers of 743 bp (Fig. 3). Virus was detected in lymphoid and non lymphoid organs of group A and B, no virus antigen was detected in control group C and D.

DISCUSSION

The present project was designed to understand the pathogenesis of virulent field IBDV through evaluating the tissue tropism. Bursa of Fabricius samples were collected and processed for IBDV isolation and identification through serological and molecular methods (Okwor *et al*., 2011). For IBDV isolation triturated bursal tissue were inoculated in embryonated eggs through chorioallantoic membrane route (Ahmad *et al*., 2005). Egg infectivity dose (EID_{50}) was estimated and infectivity titer was $10^{5.50}$/100ul.

Histopathological lesions were observed in lymphoid and non lymphoid organs of infected birds, changes were compared with the control groups. Pathological lesions were scored mild in start, followed by moderate and severe. On third day of post infection mild lymphocytic depletion and hyperplasia of epithelial cells were observed in bursa, mild hemorrhages and congestion were observed in liver, kidney and thymus. These findings are justified with findings of Zhang *et al*. (2002) and Zubeedy *et al*. (2013). On 5th day of postinfection moderate histopathological lesions were observed depleted and necrosed B lymphocytes were observed at medullar area of bursal lymphoid follicle, depleted lymphocytes were replaced by heterophils and reticuloendothelial cells. Marked leukocytic infiltration was observed in liver and spleen. Similar findings were reported by Murmu *et al*. (2014) and Tsegaye *et al*. (2014). On 9th day of postinfection marked to severe changes including severe leukocytic depletion, bursal atrophy, cyst formation and fibrous tissue proliferation were observed in bursa of Fabricius. Congestion, hemorrhages, lymphocytic necrosis, and thickening of intralobular connective tissue were seen in thymus. In kidney tubular necrosis, oedema and hemorrhages were observed. These observations were matched with findings of Rauf *et al*. (2011) and Ahmad *et al*. (2014).

Immunohistochemistry technique was used to detect the IBDV in tissue sections. Positive IBDV antigen cells

were observed as dark brown colored, finely granular, in the cytoplasm of antigen infected cells. Most of the cell associated positive staining occurred within epithelial cells (surface) of bursa and also associated with lymphocytes in lymphocytic infiltration and occasionally fibroblast and mononuclear cells were seen in different organs. The same findings were also made by Muller (1979), Zhang *et al*. (2002) and Siavosh *et al*. (2009). IBDV antigen positive cells observed in infected lymphocytes and macrophages at medullar and cortical region of lymphoid follicles of bursa and thymus. This statement was an agreement with finding of Tanimura *et al*. (1995) and Jackwood (1997). The RT-PCR was performed on lymphoid and non-lymphoid organs by using specific primers of 743bp targeted the VP2 gene of IBDV. IBDV first appeared in hematogenic organs that supplied the blood, followed by bursae. The virus reached the kidney, liver and spleen through blood after infection and enters the bursa where marked replication of virus occurred (van den Berg, 2000). RT-PCR signals were observed in bursa, thymus, spleen, kidney and liver. This statement is supported by findings of Zhang *et al*. (2002).

Conclusions: It is concluded that immunohistochemistry is better diagnostic test, to understand the mode of action of the virulent IBDV field isolate, towards the tissue tropisms of both lymphatic and non-lymphatic organs. The IBDV antigen detection rate in primary lymphoid organs was high as virus targets the lymphocytes and macrophages as compared to non lymphoid organs. RT-PCR more specifically targets the VP2 gene in IBDV infected tissues.

Authors contribution: AA and SI: Contributed in purchasing of broiler birds and kits for execution of experiment, BZ involved in conduction of experiment and samples collection, AA and BZ contributed in histopathology and immunohistochemistry, RA and HS analyzed the data, BZ and CA executed the RT-PCR. All authors interpreted the data critically revised and analyzed the manuscript for important intellectual contents and approved the final version.

REFERENCES

Abdel-Alim GA and Saif YM, 2001. Detection and persistence of infectious bursal disease virus in specific-pathogen-free and commercial broiler chickens. Avian Dis 45:646-54.

Ahmad AN, Hussain I, Siddique M, *et al.,* 2005. Adaptation of indigenous infectious bursal disease virus (IBDV) in embryonated chicken eggs. Pak Vet J 25:71-4

Ahmad AN, Iftikhar H, Masood A, *et al*., 2014. Comparative study of commercially available infectious bursal disease vaccine with egg attenuated live vaccine. Pak J Zool 46:959-66.

Ahmed H, Mostafa S, Elrefaie R, *et al*., 2015. Growth of different infectious bursal disease virus strains in cell lines from origin of lymphoid leukosis tumors. Ani Vet Sci 3:46-50.

Beenish Z, Asim A, Zafar IC, *et al*., 2017. Biochemical and histopathological changes in immune and non immune broilers after inoculation of field infectious bursal disease virus. Pak J Zool 49:1279-83.

Jackwood DJ and Nielsen CK, 1997. Detection of infectious bursal disease viruses in commercially reared chickens using the reverse transcriptase-polymerase chain reaction-restriction endonuclease assay. Avian Dis 41:137-43.

Lukert PD and Saif YM, 2004. Infectious bursal disease, In: Diseases of Poultry, 11th ed. Iowa State Press, Ames, USA pp:161-79.

Muller R, Kaufer I, Reinacher M, *et al*., 1979. Immunofluorescent studies of early virus propagation after oral infection with infectious bursal disease virus (IBDV). Zentralbl Veterinarmed B 26:345-52.

Murmu R, Islam N, Juli SN, *et al.*, 2014. Pathogenicity and immunosuppressive properties of GM-97 strain of infectious bursal disease virus in commercial broiler chickens. J Adv Vet Anim Res 1:1-7.

Nafi L, Mohd HB, Siti SA, *et al*., 2017. Adaptation and molecular characterization of two malaysian very virulent infectious bursal disease virus isolates adapted in BGM-70 cell line. Adv in Virol 10:1155-9.

Okwor EC, Eze DC, Okinkwo KE, *et al*., 2011. Comparative evaluation of agar gel precipitation test (AGPT) and indirect haemagglutination test (IHA) for the detection of antibodies against infectious bursal disease (IBD) virus in village chickens. Afric J Biotech 10:16024-7.

Raue R and Mazaheri A, 2003. Real-time RT-PCR based on genome segment B for the detection of infectious bursal disease virus. Archiv fur Geflugelkunde 67:22-7.

Rauf A, 2011. Persistence, distribution and immun opathogenesis of infectious bursal disease virus in chickens. The Ohio State University.

Razia S, Syed AH, Rahat A, *et al*., 2014.A study on prevalence of economically important viral diseases in poultry flock in district Lahore. Sci Int 26:333-5.

Reed LJ and Muench HA, 1983. A simple method of estimation fifty per cent end-point. Am J Hyg 27:493-7.

Roussn DA, Walid AS and WS Totanji, 2012. Characterization of infectious bursal disease virus field strain in Jordan using molecular techniques. Vet Arhiv 82:115-24.

Sara AM, Abdel SA and Hussein A, 2014. Molecular genotyping of the infectious Bursal disease virus (IBDV) isolated from Broiler Flocks in Egypt. Int J Vet Sci Med 2:46-52.

Siavosh HZM, Tavasoly A, Shoshtary A, *et al*., 2009. An experimental study on early pathogenesis of a very virulent isolate of infectious bursal disease virus, employing Immunohistochemistry. Iran J Vet Res 10:27.

Singh J, Banga HS, Brar RS, *et al*., 2015. Histopathological and immunohistochemical diagnosis of infectious bursal disease in poultry birds. Vet World 8:1331-9.

Subtain SM, Chaudhry ZI, Anjum AA, *et al*., 2011.Studyon pathogenesis of low pathogenic avian influenza virus H9 in broiler chickens. Pak J Zool 43: 999-1008.

Tanimura N, Tsumakoto K, Nakamura K, *et al*., 1995. Association between pathogenicity of infectious bursal disease virus and viral antigen distribution detected by immunohistochemistry. Avian Dis 39:9-20.

Tesfaheywet ZM, Hair-Bejo and Rasedee A, 2012. Biochemical Changes in Specific-Pathogen-Free Chicks Infected with Infectious Bursal Disease Virus of Malaysian Isolate. Global Veterinaria 8: 8-14.

Tsegaye K and Mersha C, 2014. Review on the Incidence and Pathology of Infectious Bursal Disease. Bri J Poul Sci 3:68-77.

Van den Berg TP, Eterradossi N, Toquin D, *et al.*, 2000. Infectious bursal disease (Gumboro disease). Rev Sci Tech 19:509-43.

Zhang MF, Huang GM and Qiao S, 2002. Early stages of infectious bursal disease virus infection in chickens detected by in situ reverse transcriptase-polymerase chain reaction. Avian Path 31:593-7

Zubeedy Al, Shamaun AA and Al-Aalim AM, 2013. Histopathological And Immune Response Against Infectious Bursal Disease In Chickens Vaccinated Against Newcastle Disease. AL-Qad J Vet Med Sci 12:66-70.