



## RESEARCH ARTICLE

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

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#### ABSTRACT

The Infectious bursal disease virus (IBDV), rapidly destroy immature B lymphocytes in bursa of fabricicus 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 3<sup>rd</sup>, 5<sup>th</sup>, 9<sup>th</sup> and 14<sup>th</sup> 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 5<sup>th</sup> day post infection until day 14<sup>th</sup>, 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 lymphocytes and macrophages. Immunohistochemistry and RT-PCR are more specific and sensitive methods for detection of IBD viral antigen.

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#### 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 lymphocytic 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 fibroplasia is 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 hypervariable 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 farms 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-allantoic 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<sup>nd</sup> passage) (Tesaheywet *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, 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  $10^{5.50}/100\mu\text{l}$ ) on 2<sup>nd</sup> 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<sup>rd</sup>, 5<sup>th</sup>, 9<sup>th</sup> and 14<sup>th</sup> 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 ten (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) test for antibodies against IBDV and found zero. The inoculum 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 transcript 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<sup>rd</sup>, 5<sup>th</sup>, 9<sup>th</sup> and 14<sup>th</sup> day of post challenge. In group A clinical signs like ruffled feathers and dullness were noticed on day 5<sup>th</sup> 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<sup>th</sup> days post infection (dpi) (Table 1).

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

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

(-) = 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, D: SAN control.

**Table 2:** Histopathological changes at 9<sup>th</sup> 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, D: SAN control.

**Table 3:** Histopathological changes at 14<sup>th</sup> 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, D: SAN control.

**Table 4:** IBD virus antigen detection in different organs through Reverse Transcriptase Polymerase Chain Reaction

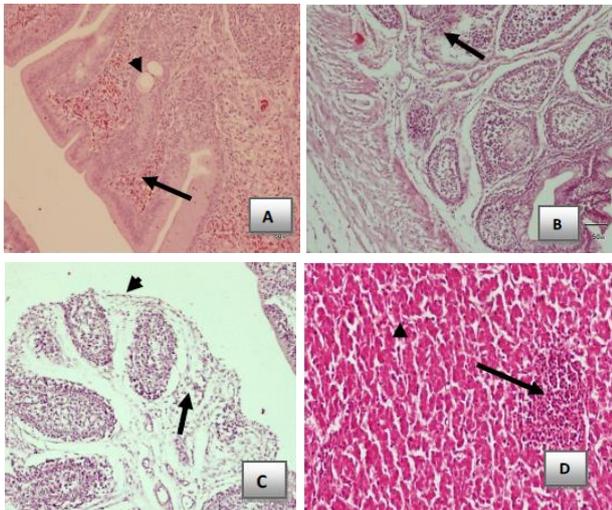
Groups	Total No. of samples	Tissues	Positive		
			on day 3 <sup>rd</sup>	on day 5 <sup>th</sup>	on day 9 <sup>th</sup>
A	6	Bursa	1	2	2
	6	Thymus	1	1	1
	6	Spleen	1	1	1
	6	Liver	0	0	0
	6	Kidney	1	1	0
	6	Lung	0	0	0
B	6	Bursa	2	2	2
	6	Thymus	1	2	1
	6	Spleen	2	2	1
	6	Liver	1	0	0
	6	Kidney	1	2	1
	6	Lung	0	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, D: SAN control.

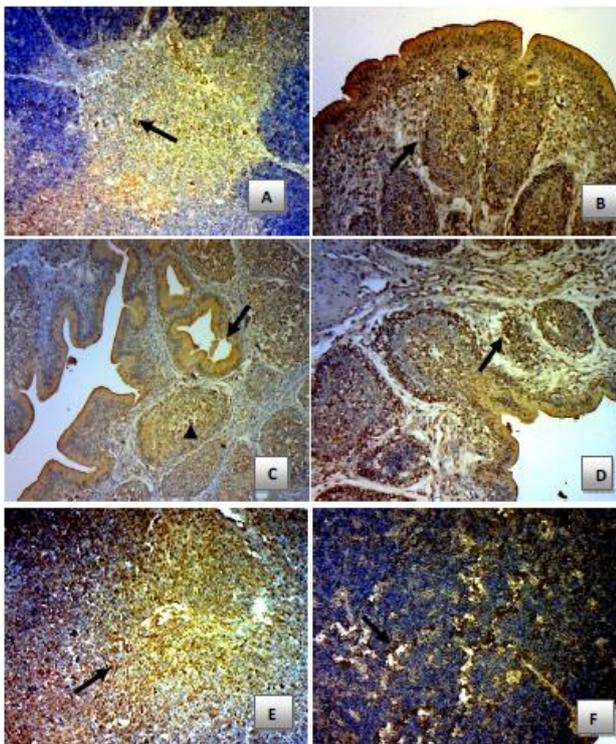
**Gross and histopathological changes:** Five birds from each group were slaughtered on 3<sup>rd</sup>, 5<sup>th</sup>, 9<sup>th</sup> and 14<sup>th</sup> days of post Infection of Infectious Bursal Disease Virus (IBDV). Gross & histopathological lesions in lymphoid and non lymphoid organs scored on different days. On 3<sup>rd</sup> day of postinfection histologically Bursal changes observed in group A were minimal, in group B mild changes observed in lymphoid organs. On 5<sup>th</sup> day postinfection Moderate histological changes observed in different organs in group A, severe changes observed in group B. Typical histological changes associated with IBDV infection, including lymphoid depletion, necrosis and hemorrhages in bursa (Fig. 1A and B). Bursal changes include follicular necrosis, necrosis of epithelial cells, cyst formation and marked fibroplasias (Fig. 1C). Severe hemorrhages and lymphocytic depletion at medullary region of thymus was observed in group B (Table 2) on day nine of post infection. Moderate changes observed on 14<sup>th</sup> day in challenged group (Table 3). Thymus showed moderate to severe changes include congestion, hemorrhages, lymphocytic necrosis at medullary region and thickening of interlobular connective tissues in challenged groups. Mild changes in liver of group B include hepatocyte necrosis and congestion (Fig. 1D). Mild lymphocytic infiltration 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 3<sup>rd</sup> to 14<sup>th</sup> 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 3<sup>rd</sup> day post infection until day 14<sup>th</sup>, 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 5<sup>th</sup> post infection, antigen was detected through Immunohistochemistry in medullary 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 9<sup>th</sup> 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 14<sup>th</sup> 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.

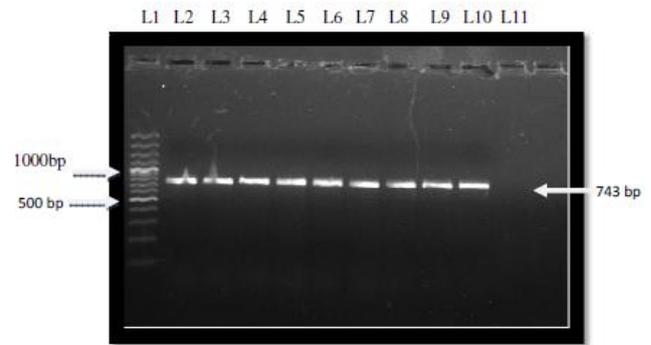
IBD virus antigen was detected in lymphoid organs of Bursa, Thymus and Spleen and non-Lymphoid organs of Kidney, Liver and Lung on 3, 5 & 9 day post infection through Reverse Transcriptase Polymerase Chain Reaction (Table 4).



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



**Fig. 2:** **A:** IHC staining of Thymus from group A at 5<sup>th</sup> day PI. Antigen mainly observed in the medullar region of lymphoid follicle. **B:** IHC staining of Bursa of Fabricius from group B at 5<sup>th</sup> 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 9<sup>th</sup> 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 9<sup>th</sup> 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 5<sup>th</sup> day PI. Antigen observed in degenerated lymphoid cells around central artery. **F:** IHC staining of Thymus from group B at 3<sup>rd</sup> day of PI. Antigen mainly observed in the thymus lymphoid follicles at central region.



**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<sub>50</sub>) was estimated and infectivity titer was  $10^{5.50}/100\text{ul}$ .

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 observed in bursa, mild hemorrhages and congestion was observed in liver, kidney and thymus. These findings are justified with findings of (Zhang *et al.*, 2002; Zubeedy *et al.*, 2013). On 5<sup>th</sup> day of postinfection moderate histopathological lesions observed depleted and necrosed B lymphocytes observed at medullar area of bursal lymphoid follicle, depleted lymphocytes replaced by heterophils and reticuloendothelial cells. Marked leukocytic infiltration was observed in liver and spleen. Similar findings reported by (Murmu *et al.*, 2014; Tsegaye *et al.*, 2014). On 9<sup>th</sup> day of postinfection marked to severe changes including severe leukocytic depletion, bursal atrophy, cyst formation and fibrous tissue proliferation 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 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 seen in different organs. The same findings were also made by Siavosh *et al.* (2009), Zhang *et al.* (2002) and Muller (1979). 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 reverse transcriptase polymerase chain reaction (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.

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