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Original Article

Evaluation of viral load and expression level of apoptotic genes in selected tissues of two hybrids of commercial broiler chickens challenged with infectious bronchitis virus: a comparative study

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Abstract

Background: Infectious bronchitis virus (IBV) causes severe economic losses worldwide. IBV has a broad tissue distribution with different viral loads in different tissues. Additionally, IBV can induce apoptosis in infected cells. **Aims:** The present study aimed to evaluate the role of the genetic background of chickens in viral load and the expression level of apoptotic genes in different tissues of two hybrids of commercial broiler chickens (Ross 308 and Cobb 500) challenged with IBV. **Methods:** Chickens at 21 days of age were nasally challenged with 200 µL of allantoic fluid containing 10^4 EID_{50}/ml of Iranian variant-2-like IBV (IS/1494). The expression level of apoptotic genes (*Fas*, *FasL*, *Bax*, and *Bcl-2*) in the tracheal and renal tissues and the amount of viral load in the tracheal, renal, and cloacal swab samples were investigated two, five, and seven days after IBV infection by RT-qPCR assay. **Results:** The amount of viral load and apoptotic the expression level of apoptotic genes in the tracheal (two and five days after infection) and renal samples (seven days after infection) were significantly higher in the Ross challenged group than in the Cobb challenged group. Furthermore, no difference was observed in the cloaca viral load on sampling days. **Conclusion:** To our knowledge, this is the first report that evaluated the role of the chickens' genetic background in the amount of viral load and the expression level of apoptotic genes against IBV. Further studies are needed to investigate the pathogenic characteristics of IBV in Ross 308 and Cobb 500 chickens.

Key words: Apoptosis, Broiler chickens, Infectious bronchitis virus, Viral load

Introduction

Infectious bronchitis virus (IBV) is an acute and highly infectious disease in chickens, leading to dire economic consequences locally and globally (Liu *et al*., 2017; Barjesteh *et al*., 2020). IBV (a single-stranded positive-sense, enveloped RNA virus of 27-32 kb length) belongs to the family Coronaviridae (Bande *et al*., 2016). Various variants of IBV have been associated with renal, respiratory, and reproductive diseases in chickens of different ages (Ganapathy *et al*., 2013).

IBV is a fast-replicating, highly mutable virus with great recombination capacity, leading to the formation of several strains (De Wit *et al*., 2018; Marandino *et al*., 2019). One of the current effective prevention measures for this disease is vaccination. Although vaccination has been effective, curbing the spread of the disease is not straightforward due to strain diversity and lack of any complete cross-protection between them (Jordan, 2017; De Wit *et al*., 2018; Marandino *et al*., 2019).

The respiratory tract is the first route of infection in most IBV strains (Najimudeen *et al*., 2020). IBV attaches to the respiratory epithelium using the α2, 3-linked sialic acid receptor, which results in virus replication (Winter *et al*., 2006; Madu *et al*., 2007). Additionally, other organs could be infected by viremia following infection of the respiratory tract with IBV (Najimudeen *et al*., 2020).

The innate immune system is the first line of defense against IBV. One of the components of this system is apoptosis, which plays non-specific protective roles (Chhabra *et al*., 2016). Apoptosis, originating from the activation of intracellular self-demolition biochemical events, is a method of programmed cell death that occurs in multicellular organisms (Elmore, 2007; Ampomah and Lim, 2020). The relationship between apoptosis and viral diseases is complex. Induction of apoptosis in infected cells has an important role in triggering the host's antiviral reaction and can directly interfere with viral replication. Nonetheless, several viruses actively trigger

this action to help their replication (Clarke and Tyler, 2009).

Previous studies have revealed that IBV has a broad tissue distribution and different viral loads in tissues (Najafi *et al*., 2016a; Ren *et al*., 2020). Additionally, several studies have demonstrated that IBV induces apoptosis (Liu *et al*., 2001; Zhong *et al*., 2012; Chhabra *et al*., 2016; Liu *et al*., 2017). However, there is limited information about the evaluation of viral load and the expression level of apoptotic genes in different hybrids of chickens challenged with IBV. Therefore, further studies are needed to investigate the role of the genetic background of chickens in IBV. In the present study, we assessed the viral load in the tracheal, renal, and cloacal swab samples and the expression levels of apoptotic genes in the tracheal and renal tissues taken from Ross 308 and Cobb 500 challenged with Iranian variant-2-like IBV (IS/1494).

Materials and Methods

Experimental design

The current study was conducted as a randomized controlled experimental study. Chickens were selected from two breeds (Ross 308 and Cobb 500). A total of 160 one-day-old broiler chicks (80 Ross and 80 Cobb) were randomly divided into four groups and separately placed in two rooms (hereafter named the challenged group room and control group room). Our four groups were as follows: the Cobb control group, Ross control group, Cobb challenged group, and Ross challenged group. Each group received water and food separately.

We labeled all chickens placed in the rooms (C for Cobb and R for Ross). To ignore the role of maternal antibodies in creating protection against infectious bronchitis, enzyme-linked immunosorbent assay (ELISA) was performed at 21 days of age to measure anti-IBV antibody titers (IDEXX Kit, USA), following the manufacturer's instructions. For the purpose of this study, only chickens with negative antibody titers were included. At 21 days of age, all the challenged and control groups were nasally challenged with 200 µL of allantoic fluid containing 10^4 50% egg infective dose (EID_{50})/ml of the variant-2-like strain (IS/1494) and 0.2 ml of sterile phosphate-buffered saline (PBS), respectively.

Strain history

Iranian variant-2-like IBV (IS/1494; accession number: KT583601; Najafi *et al*., 2016a) was used in the study. In addition, the Reed-Muench method was used to calculate EID_{50} (Reed and Muench, 1938).

Sample collection and preparation

Ten chickens were sampled from groups two, five, and seven days after infection. The chickens were then euthanized using carbon dioxide and cervical vertebra dislocation, as required by the University of Tehran's ethical standards. Tracheal, renal, and cloacal swab samples were immediately taken, all in sterile conditions. In addition, the tracheal and renal tissues were biopsied and tested for apoptotic gene expression. Finally, the tracheal, renal, and cloacal swab samples were used for virus detection and viral load measurement.

Real-time polymerase chain reaction

RNA extraction and complementary DNA synthesis

Total RNA was extracted from tissue and swab samples using the SinaPure RNA Kit (SinaClon, Iran), and then treated with deoxyribonuclease I (DNase I). The quality and quantity of total RNA extracted from tissues and swabs were determined using 230, 260, and 280 nm spectroscopic measurements. RNA samples with an absorbance ratio of 1.9-2.2 at OD260/280 and greater than 2 at OD260/230 were utilized for complementary DNA (cDNA) synthesis. Single-stranded cDNA was synthesized from 2 µg total RNA using reverse transcriptase and random hexamer primer. To do so, we used the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Canada), following the manufacturer's instructions.

Real-time polymerase chain reaction for the evaluation of apoptotic genes expression

The cDNA resulting from renal and tracheal tissues evaluated apoptotic gene expression. Table 1 lists primer pair sets of apoptosis-regulating genes and 28S ribosomal RNA (rRNA, as a housekeeping gene; Yuan *et al*., 2016). For quantitative reverse transcription-polymerase chain reaction (RT-PCR), the 25 µL real-time PCR composition contained 2.5 µL cDNA, 12.5 µL SYBR Premix (SinaClon, Iran), 0.5 µL forward and 0.5 µL reverse primer at a final concentration of 0.10 µm, and 9 µL ribonuclease (RNase)-free water. The reaction condition was carried out at 95°C for 3 min, then 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s using a Corbett Life Science Rotor-Gene 6000 Cycler. Expression fold changes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1: A list of oligonucleotides primers used in the real-time PCR assays

Genes	Primer (5´-3´)	Amplicon size (bp)
Fas	F: TCCACCTGCTCCTCGTCATT R: GTGCAGTGTGTGTGGGAACT	78
FasL	F: GGCATTCAGTACCGTGACCA R: CCGGAAGAGCACATTGGAGT	77
Bax	F: GGTGACAGGGATCGTCACAG R: TAGGCCAGGAACAGGGTGAA	108
Bcl-2	F: TGTTTCTCAAACCAGACACCAA R: CAGTAGGCACCTGTGAGATCG	198
28srRNA	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	61
5´UTR-IBV	F: GCTTTTGAGCCTAGCGTT R: GCCATGTTGTCACTGTCTATTG	143

Real-time PCR for IBV titration

The resulting cDNAs from the renal, tracheal, and cloacal swabs were used for IBV titration. In addition,

real-time PCR was employed to amplify the conserved sequence within the 5´ untranslated region (5´ UTR) of the IBV genome using the primer pairs listed in Table 1 (Callison *et al*., 2006). The 20 µL real-time PCR contained 2 µL cDNA, 10 µL SYBR Premix (SinaClon, Iran), 0.4 µL forward and 0.4 µL reverse primers at a final concentration of 0.10 µm, and 7.2 µL RNase-free water. The reaction was performed using a Corbett Life Science Rotor-Gene 6000 Cycler. The PCR cycling parameters included 95°C for 3 min, followed by 44 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s (Najafi *et al*., 2016b).

Statistical analysis

On each day of the study, the t-test (independent two-sample t-test) was used to compare viral load and the expression level of apoptotic genes between the two challenged groups (Ross and Cobb challenged groups). All analyses were performed at a significance level of 5%. Stata 14 (StataCorp, College Station, Texas, USA) was used for data analysis.

Results

Three chickens (Ross breed) were excluded from the study due to the positive anti-IBV antibody titer at 21 days of age. No positive antibody titer was found for Cobb chickens at this age.

Findings of apoptotic gene expression in tracheal and renal tissues

The expression level of apoptotic genes in the tracheal and renal tissues was calculated two, five, and seven days after infection in the Cobb and Ross challenged groups and compared with the Cobb and Ross control groups. The results showed that viral challenge with Iranian variant-2-like IBV (IS/1494) induced apoptosis in renal and tracheal tissues. Furthermore, induction of apoptotic gene expression was not observed in the control groups. Moreover, the expression levels of apoptotic genes were different in the tracheal and renal tissues of these two breeds in the three sampling situations. We also observed that the expression level of apoptotic genes in the tracheal tissue of the Ross challenged group two and five days after infection was significantly higher compared to the Cobb challenged group. The comparison of the expression level of apoptotic genes in renal tissues showed that the expression level of apoptotic genes for the Ross challenged group seven days after infection was significantly higher than that for the Cobb challenged group (for more details, see Tables 2 and 3).

Table 2: The mean expression level of apoptotic genes in the tracheal samples of the challenged groups and statistical analysis (the numbers in parentheses indicate SD)

Apoptotic gene	Breed	2 days post-infection	5 days post-infection	7 days post-infection
Fas	Cobb	20.20 (2.97)	22.10 (3.21)	20.00 (2.90)
	Ross	28.80 (2.25)	31.00 (3.65)	21.70 (1.50)
		t=7.29, P<0.0001	t=5.78, P<0.0001	t=1.65, P=0.11
FasL	Cobb	4.40 (1.34)	5.80 (1.31)	4.90 (1.52)
	Ross	7.90 (1.10)	10.10 (1.37)	5.20 (0. 91)
		t=6.35, P<0.0001	t=7.15, P<0.0001	t=0.53, P=0.60
Bax	Cobb	7.60 (1.57)	7.60 (1.57)	7.52 (0. 82)
	Ross	11.70 (0.94)	12.00 (1.76)	7.60 (0. 52)
		t=7.04, P<0.0001	t=5.87, P<0.0001	t=0.26, P=0.80
Bcl-2	Cobb	3.40 (0. 84)	4.30 (1.56)	3.50 (0.70)
	Ross	4.90 (1.10)	7.40 (0.84)	3.90 (1.44)
		t=3.42, P=0.003	t=5.50, P<0.0001	t=0.78, P=0.44

Table 3: The mean expression level of apoptotic genes in the renal samples of the challenged groups and statistical analysis (the numbers in parentheses indicate SD)

Apoptotic gene	Breed	2 days post-infection	5 days post-infection	7 days post-infection
Fas	Cobb	1.40 (0.51)	2.60 (0.69)	2.90 (0.56)
	Ross	1.50 (0.70)	2.80 (0.63)	4.0 (0.66)
		t=0.36, P<0.72	t=0.67, P=0.51	t=3.97, P=0.0009
FasL	Cobb	1.40 (0.51)	3.70 (0.67)	2.20 (0.42)
	Ross	1.50 (0.52)	3.80 (0.63)	3.90 (0.56)
		t=0.42, P=0.67	t=0.34, P=0.73	t=7.69, P<0.0001
Bax	Cobb	1.50 (0.52)	2.70 (0.67)	3.20 (0.63)
	Ross	1.60 (0.69)	2.90 (0.56)	4.80 (0.63)
		t=0.36, P=0.72	t=0.71, P=0.48	t=5.65, P<0.0001
Bcl-2	Cobb	1.40 (0.51)	2.70 (0.67)	2.90 (0.56)
	Ross	1.50 (0.52)	2.90 (0.56)	3.90 (0.73)
		t=0.42, P=0.67	t=0.71, P=0.48	t=3.39, P=0.003

Findings of viral load in the tracheal, renal, and cloacal swabs

Renal, tracheal, and cloacal viral loads were measured two, five, and seven days after infection for all challenged and control groups. Viral loads in all samples of the control groups were negative. However, the viral load was detectable in all swab samples of the challenged groups; the highest viral load was observed in the tracheal and cloacal samples taken five days after infection. In addition, we observed the viral load in renal swabs that increased gradually and peaked at seven days after infection.

Statistical analysis showed that two and five days after infection, the mean viral load in the Ross challenged group's tracheal samples was significantly higher than the Cobb challenged group. Additionally, seven days after infection, the mean viral load in the Ross challenged group's renal samples was significantly higher than the Cobb challenged group. Furthermore, no significant difference was observed between the viral loads of the cloaca samples in the challenged groups (for more details, see Table 4).

Table 4: The logarithm base 2 mean viral load in the samples of the challenged groups and statistical analysis (the numbers in parentheses indicate SD)

Days post-infection	Trachea	Kidney	Cloaca
2	Cobb: 5.02 (0.65) Ross: 7.25 (0.74) t=7.12, P<0.0001	0.42 (1.33) 0.66 (2.09) t=0.30, P=0.76	1.71 (2.77) 3.16 (4.09) t=0.92, P=0.36
5	Cobb: 6.73 (0.22) Ross: 7.43 (0.37) t=5.03, P=0.0001	3.37 (2.96) 3.28 (3.46) t=0.06, P=0.95	7.28 (0.88) 7.31 (0.49) t=0.09, P=0.92
7	Cobb: 6.05 (0.70) Ross: 6.46 (0.65) t=1.38, P=0.18	4.93 (0.46) 5.99 (0.83) t=3.48, P=0.002	5.08 (2.70) 6.68 (0.52) t=1.83, P=0.08

Discussion

The present study aimed to evaluate the amount of viral load in the tracheal, renal, and cloacal swab samples and the expression levels of apoptotic genes in the tracheal and renal tissues of Ross 308 and Cobb 500 breeds challenged with Iranian variant-2-like IBV (IS/1494) two, five, and seven days after infection.

Like most respiratory diseases, the respiratory tract is the first route of infection in most IBV strains (Najimudeen *et al*., 2020), and other organs could be infected by viremia following infection of the respiratory tract with IBV (Najimudeen *et al*., 2020). Consistent with Najimudeen *et al*.'s study (2020), the virus was observed in all investigated samples (tracheal, renal, and cloacal swabs) from the challenged groups during all sampling conditions. A similar finding was reported by Najafi *et al*. (2016a), who showed that Iranian variant-2-like IBV (IS/1494) had a broad tissue distribution and was detectable in the respiratory tract, digestive system, and renal tissues. The current study's findings indicated that the viral load in the tracheal and cloacal samples was higher than that of renal ones. This is in line with Ren *et al*.'s study (2020), which found that QX-like strains had a higher viral load in the trachea than the kidney. Also, it has been reported that the 02 serotype could show a higher viral antigen in the nasal turbinate compared to the kidneys of young chicks (Dolz *et al*., 2012). In the current study, we observed that two and five days after infection, viral loads in the Ross tracheal swabs were significantly higher than those in the Cobb tracheal swabs on the same days.

Furthermore, seven days after infection, viral loads in the Ross renal swabs were significantly higher than those in the Cobb renal swabs on the same day. Since the α2, 3-linked sialic acid receptor-mediated IBV binding and infection of host cells (Winter *et al*., 2006; Madu *et al*., 2007), it is likely that the expression level of the α2, 3-linked sialic acid receptor differed in the epithelium of the tracheal and renal tissues in Cobb and Ross breeds. However, further studies are needed to confirm this viewpoint.

Additionally, Belkasmi *et al*. (2020) and Zegpi *et al*. (2020) found that viral load was associated with histological lesions. Since the amount of viral load in the samples taken from the challenged groups of both breeds was different, the authors suspect that pathological lesions may also be different in the two breeds, and this could be considered one of the potential areas for future research.

In this study, we found that viral challenge with Iranian variant-2-like IBV (IS/1494) induced the expression of apoptotic genes in the kidney and trachea of both challenged groups. Similar to our findings, several studies have revealed that coronaviruses can induce apoptosis. For instance, Zhao *et al*. (2006), Ding *et al*. (2012), Lan *et al*. (2013), and Chhabra *et al*. (2016) demonstrated that coronaviruses induced apoptosis in various infected cell types. In addition, several studies have found that some viruses are involved in cell apoptosis mediated by *Fas*/*FasL* signaling as a response to viral infections, including hepatitis C virus (HCV; Chen *et al*., 2011) and dengue virus (DENV; Liao *et al*., 2010). Several viruses can stimulate the process of cell apoptosis via regulating the levels of apoptosis regulator *Bcl-2* family protein members, including severe acute respiratory syndrome (SARS) coronavirus (Tan *et al*., 2007) and Epstein-Barr virus (EBV; Fu *et al*., 2013).

Furthermore, it has recently been reported that the expression levels of genes involved in apoptosis in chicken embryo kidney cells and tracheal epithelial cells were upregulated during the infection of virulent IBVs (Chhabra *et al*., 2016; Liu *et al*., 2017). In addition, evidence shows that IBV could stimulate apoptosis via caspase-dependent (Liu *et al*., 2001) and intrinsic-dependent pathways adjusted through *Bcl-2* proteins in Vero cells (Zhong *et al*., 2012). Moreover, evidence indicates that IBV could induce apoptosis through the extrinsic pathway regulated by *Fas*/*FasL* (Han *et al*., 2017).

Cell apoptosis induced by IBV has also been demonstrated in Vero cells, DF1 cells, and chicken

embryo kidney cells (Zhong *et al*., 2012; Chhabra *et al*., 2016). In the present study, the expression levels of apoptotic genes were different in the tracheal and renal tissues of these two breeds on the three sampling days. Furthermore, in both breeds, it was observed that the expression level of apoptotic genes was more in the tracheal tissues compared to the renal tissues. In addition, it was observed that the expression level of apoptotic genes in the investigated tissues of the Ross challenged group was often higher than that of the Cobb challenged group.

Previous studies have reported that the cell apoptosis induced by the virus is associated with tissue damage, where the apoptosis of infected cells can interfere with viral replication (Clarke and Tyler, 2009). Since the expression level of apoptotic genes in the investigated tissues of the Ross challenged group was often higher than that of the Cobb challenged group, the authors suspect that pathological lesions may be different in the two breeds, and this could be considered as one of the potential areas for future research.

In conclusion, the study was conducted to evaluate the role of the genetic background of chickens in the amount of viral load and the expression level of apoptotic genes in different tissues of two hybrids of commercial broiler chickens (Ross 308 and Cobb 500) challenged with IBV. The results showed that the amount of viral load and the expression level of apoptotic genes in the tracheal (two and five days after infection) and renal samples (seven days after infection) were significantly higher in the Ross challenged group than the Cobb challenged group. Furthermore, no difference was observed in the cloaca viral load on sampling days.

To our knowledge, this is the first report that evaluated the role of the genetic background of chickens in the amount of viral load, and the expression levels of apoptotic genes in different tissues of two hybrids of commercial broiler chickens (Ross 308 and Cobb 500) challenged with IBV. We observed that the expression level of apoptotic genes and the amount of viral load could be affected by breed. Further studies are needed to investigate the pathogenic characteristics of IBV in Ross 308 and Cobb 500 chickens.

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Conflict of interest

The authors declare no conflict of interest.

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