

Viral quantity and pathological changes in broilers experimentally infected by IRFIBV32 isolate of infectious bronchitis virus

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Abstract An Iranian isolate of avian infectious bronchitis virus IRFIBV32 was quantified in experimentally infected broilers using real-time reverse transcriptase polymerase chain reaction and histopathological changes was investigated. Thirty-six 3-week-old commercial broilers were inoculated by 10^5 ELD₅₀/0.1 ml of the virus. On the various days post inoculation (dpi) different tissues were collected. The virus strongly started the replication in trachea at 1 dpi and reached to the maximum titer at 3 dpi. The highest IBV RNA level was shown in this organ. In lung, the virus was replicated with the titer lower than that of the trachea, but it rose up more at 5 dpi. The kidneys were the tissues with the least viral genome copy number, although the duration of the virus presence was considerable. The virus replicated in testes sooner than ovaries also disappeared sooner but the maximum viral yield in the ovaries was more. The virus titer in the studied tissues had an interesting fluctuation especially in caecal tonsils. Testes and ovaries were the organs that the virus could reactivate without using any chemical. The most severe lesions were observed in tracheae but they appeared in the

lungs later. Lymphocyte infiltration in the kidneys was noted from 5 dpi even sooner than the lungs. There were no lesions in the caecal tonsils, testes and ovaries in spite of the virus replication in a high titer.

Keywords Chicken · IBV · Real time RT PCR · Quantitation · Pathology

Infectious bronchitis (IB) is an acute, highly contagious upper respiratory disease of chickens. IB is characterized by tracheal rales, sneezing and coughing, increased oculo-nasal secretion and excess mucus in the trachea accompanied by decreases in both weight gain and feed efficiency. Economic consequences to the poultry industry comprise growth retardation and high morbidity and mortality rates in meat-type birds. In addition, reduced egg production, decreased internal, external egg quality, hatchability and fertility have been documented in layers and breeders [8]. This disease is caused by infectious bronchitis virus (IBV), a member of the family Coronaviridae (order Nidovirales) and genus Coronavirus [6]. IBV has been shown to replicate in many respiratory tissues including trachea, lungs, and air sacs, causing respiratory infection; in some urogenital tissues such as kidney, testes and oviduct, causing minor or major nephritis; and in many parts of the alimentary tract as well [12]. Live attenuated and inactivated vaccines have been developed to control IB. Despite extensive vaccination, IB has been a perpetual problem in the Iranian poultry industry since the disease was first reported in 1994 [2, 18]. A new isolate of IB virus (IRFIBV32) from broiler chicken in Fars province, Southern Iran, is isolated and identified by Boroomand et al. [6]. Furthermore, the tissue distribution and clinical signs of experimentally infected chicks by this isolate are also studied [5].

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Currently, Real time reverse transcription polymerase chain reaction (RRT PCR) technique has been developed and used widely for rapid, sensitive and accurate diagnosis of different diseases. A RRT PCR test has been developed using a Taqman probe for the detection of American strains of IBV from tracheal swabs [7] and a locked nucleic acid (LNA) probe-based RRT PCR assay for the detection of the Australian IBV strains [10]. Jackwood et al. [14] used a RRT PCR diagnostic test with the use of fluorescence resonance energy transfer (FRET) technology to detect several common strains of IBV. In this study, we identified IBV load in different tissues of experimentally infected broilers to clarify the replication strength of IRFIBV32 Isolate at intervals post challenge. This was achieved using TaqMan[®]-labeled probe two-step RRT PCR assay targeting the N gene of the virus. The N gene was chosen since it is highly conserved among IBV strains. We believe that the quantitation of the virus in different organs of the infected bird at different times post virus inoculation accompanied with pathological study may further help us to investigate the virus pathogenesis.

The virus isolate used in this study was IRFIBV32 (GenBank: HQ123359.1). Some characteristics of the isolate reported previously [6]. Virus propagation was performed in 10-day-old embryonated chicken eggs, as described previously [17]. The embryo lethal dose (ELD₅₀) of infected allantoic fluid was calculated according to the Reed and Muench [17] method. In the present study, allantoic fluid containing 10⁵ ELD₅₀/0.1 ml of the virus was used to induce the infection. Fifty-nine apparently healthy 1-day-old Ross broiler chicks were divided randomly into two experimental groups comprising group 1 (challenged group: n = 41) and group 2 (control group: n = 18). Two groups of chicks were reared separately in the Animal Research Unit of the Veterinary School of Shiraz University and received feed and water ad libitum during the experiment. All experiments were conducted after institutional approval of the animal use committee of Shiraz University. Prior to challenge, five birds from the experimental group (group 1) were killed and their organs were investigated for IBV detection. At 20 days old, all birds in group 1 was challenged intranasally with 100 µl allantoic fluid containing 10⁵ ELD₅₀ of the virus. The chicks in group 2 kept as unchallenged control. All the chicks were monitored daily for 20 days for clinical signs, and mortality. On 1, 2, 3, 5, 7, 11, 13, 15 and 20 days post inoculation (dpi) four birds from the challenged group and two birds from the control group were randomly selected. Then, they were bled for plasma separation and sacrificed for collecting tissue samples. Gross lesions were recorded and their trachea, lungs, kidneys, caecal tonsils, testes and ovaries were aseptically collected separately for the virus detection, titration and histopathological evaluations. All

tissue samples were immediately frozen and stored at -70 °C until needed for the RNA extraction. The samples for histopathological evaluations were fixed in 10 % neutral buffered formalin, embedded in paraffin and the sections were stained with haematoxylin and eosin and studied by an ordinary light microscope. RNA extraction from clinical samples was performed with the AccuzolTM (Bio-Neer Corporation, South Korea) according to the manufacturer's protocol. Appropriate amount of plasma (250 µl) or homogenized tissues (50–100 mg) were used for the extraction. Isolated RNAs were solved in a final volume of 50 µl distilled water (DW) and was quantified by spectrophotometry. Appropriate RNase Inhibitor and DNase (Fermentas) were added and stored at -70 °C until assayed. For standard preparation, 100 µl infected allantoic fluid was used for RNA extraction and cDNA synthesis was carried out using BIONEER RT-premix kit according to the manufacturer's instructions. PCR was performed to amplify 276 bp fragment of N protein gene of IBV that included in the RRT PCR assay amplicons [1]. Five microliter of total RNA and 10 pmol of antisense primer were used for cDNA preparation. Three microliter of cDNA were added to 22 µl PCR master mix containing: 2.5 µl 10X PCR buffer, 0.75 µl MgCl₂ (50 mM), 0.5 µl dNTPs (10 mM), 10 pmol of each primer, 15.75 µl DNase/RNase free DW and 0.5 µl Taq DNA Polymerase (5 IU/µl). The program in Ependorf thermal cycler was 95 °C for 3 min and 35 cycles including: 95 °C for 45 s, 55.6 °C for 45 s, 72 °C for 50 s and a final extension step at 72 °C for 7 min. The PCR products were separated in a 1 % (w/v) agarose gel and visualized under ultraviolet light after staining with ethidium bromide. PCR products (276 bp) was cloned into a T-vector (pTZ57R/T) based on Fermentas I nsTAcloneTMPCR cloning kit instructions. The plasmid DNA was amplified in *E. coli* strain GM2163 and purified using BIONEER Accuprep Plasmid Extraction kit. The recombinant plasmid was verified by DNA sequencing. The concentration of the plasmid DNA was calculated using spectrophotometry. Recombinant plasmids were serially diluted tenfold as a standard in the RRT PCR. The cDNA synthesis from normalized RNA of the samples was carried out and then RRT PCR was performed using a mixture of 20 pmol random hexamer and 20 pmol of reverse primer [1]. The RRT PCR primers and TaqMan probe used in this study were described previously by Chousalkar et al. [10]. The assays were performed in the BIO-RAD MiniOpticonTM System. The reaction mixture contained 5 µl of target cDNA, 1 µl of each primer and probe at concentration of 10 pmol/µl, 10 µl of 2x master mixes, 0.2 µl of UNG (Uracil-N-Glycosylase), and 1.8 µl of DW in a final volume of 20 µl. UNG treatment and two step cycles program were as previous [10]. Viral RNA copy numbers (expressed as copies per 1 µg of total RNA)

were quantified by comparison with a tenfold serially diluted recombinant plasmid standard of known concentration.

The quantity of the virus RNA in various organs of the challenged birds at the different dpi was determined by RRT PCR. All organs of the five birds killed at 0 dpi (prior to challenge) were negative for viral RNA. The results showed that the plasma samples of the challenged chickens were negative for the virus in any times post inoculation. The load of the virus in various tissues after challenge was shown in Table 1. The virus had its highest load in trachea on 3 dpi meanwhile lungs had the maximum level of the virus on 5 dpi. The virus existed at both organs up to 13 dpi. It was interesting that the titers on 13 dpi were more than those on 11 dpi. Although the viral titer in kidneys was lower than the other tissues, the duration of virus replication was considerable (up to 15 dpi). The maximum viral load in this organ was on 7 dpi. There was no any evidence for virus replication in the ovary up to 3 dpi but it was appeared on 5 dpi with the highest level. Although the virus was absent on 15 dpi, it was seen on 20 dpi again. Testes were the organs that the virus replicated very soon and it was continued till 11 dpi with the highest level on 3 dpi. Caecal tonsils were the tissues with the highest duration of the virus replication and a considerable amount of the viral RNA. The maximum yield was seen on 5 dpi. It was the unique tissue that not only viral replication extended in all days but also there was an interesting fluctuation in the viral titer in the different days. The quantity of the viral RNA was remarkable even in the last day of the assay (Table 1). No virus was detected in samples from unchallenged birds, indicating that biosecurity precautions were adequate.

Histopathological examination of the tracheal sections showed loss of cilia, and degenerative changes in the epithelial cells, decreased goblet cells and mucus glands

with infiltration of plasma cells and lymphocytes into the lamina propria from 1 to 9 dpi. On 11, 15 and 20 dpi, no changes were recorded in sections of trachea. Infiltration of mononuclear cells and interstitial pneumonia was evident in these chicks on 7 and 9 dpi. The kidney lesions consisted of infiltration of lymphocytes in the interstitial space. The lesions were seen from 5 to 20 dpi. There were no lesions in the caecal tonsils, testes and oviducts. Also no lesions were found in the tissue sections of the control animals.

IBV infection in chickens is known to be initiated by replication in the upper respiratory tract, which is usually restricted to the ciliated and mucus secreting cells of the nostrils, Harderian gland, trachea, lungs, and air sacs. Different type of IBV shows different tissue tropisms [5]. The maximum amount of viral RNA in the trachea was observed on 3 dpi, while that for lung was at 5 dpi. The viral RNA in trachea was not detected at 15 dpi therefore; the tracheal epithelium can support a finite amount of the virus. It is generally believed that upper respiratory tract is the primary site of IBV infection, although IBV can also replicate in the reproductive, renal, and digestive systems [8]. Callison et al. [7] detected the maximal load of viral RNA in the trachea at 5 dpi. Mahdavi et al. [15] detected increasingly viral antigen in the lung and tracheal epithelium cells from 2 to 5 dpi. Grgić et al. [13] found that histological lesions were very prominent in the trachea during the first 6–7 days after exposure to IBV while, in the present study, tracheal lesions were seen from 1 to 7 dpi. In histopathological examination, tissue lesions including loss of cilia and degenerative changes in the epithelial cells, decreased mucus glands and mononuclear cells infiltration in sections of trachea, interstitial pneumonia and interstitial nephritis are consistent with previous studies [10]. As the isolate IRFIBV32 was able to replicate in trachea for a longer duration, it may be a reason for severity of its virulence.

Table 1 Copy numbers of IBV genome per 1 µg of total RNA in different infected tissues of chickens at different dpi

dpi ^a	Tissues					
	Trachea	Lung	Kidney	Ovary	Testis	Caecal tonsils
1	221740 ^b	110155	20667	0	1548	2707070
2	1045981	467016	23380	0	807889	1339068
3	10878619	789780	273617	0	1741502	4985837 ^c
5	2388166	2648971	299412	2232480	115834	9278220
7	811372	1274234	320741	380612	0	2043642
11	18894	63984	45069	274863	81326 ^c	6973352 ^c
13	109066 ^c	83666 ^c	2400	2937	0	2295132
15	0	0	4762 ^c	0	0	1713176
20	0	0	0	148762 ^c	0	3784095 ^c

^a Days post inoculation

^b Viral RNA copy number

^c Increasing of viral RNA after decreasing

In this study urinary tract showed marked infection from 1 to 15 dpi and the maximal level of the virus was found on 7 dpi. All kidneys sampled on 20 dpi lacked the viral RNA but the kidney lesions were seen until 20 dpi. Comparison of presence pattern of the virus in kidneys, to trachea and lung showed that presence of the virus more prolonged in the kidneys. These data indicate that IRFIBV32 has nephrotropic properties as many strains of IBV [15]. The results showed that the ovary can support active replication of the IRFIBV32 isolate of IBV between 5 and 13 dpi and virus load appears to peak at 5 dpi. Chousalkar et al. [9] detected T strain of IBV in oviduct from 6 dpi and the viral load appeared to peak between 10 and 14 dpi. IBV RNAs were detected in testes between 1 and 11 dpi. Although the viral load in the ovaries was more than testes, the virus replication in the ovaries was overdue in comparison to the testes. Detection of IBV RNA in the testes corroborates findings by others that IBV replicates in the male testicles [11]. In spite of the virus replication in male and female reproductive system, there were no lesions in these organs.

IBV Infection of enteric tissues does usually not manifest clinically but persists for long periods and results in fecal virus shedding. We detected the viral RNA in the caecal tonsils of infected chicken from 1 to 20 dpi and the maximal loads of the virus were on 5 dpi. Comparison of detection of the virus from the caecal tonsils and the other organs showed that the IRFIBV32 isolate can replicate and be detected in the caecal tonsils for a longer time. Naqi et al. [16] isolated Massachusetts serotype vaccine virus from cloacal swabs at 63 dpi. Mahdavi et al. [15] detected the viral antigens in intestine and kidney epithelial cells from 3 to 5 dpi. The viral RNA was not detected in the blood of inoculated chickens in this study. No virus was detected in any tissue of the unchallenged group.

The broad distribution of 2, 3-linked sialic acid in different organs explains the pan-tropic nature of IBV in chicken, but it cannot be the only determinant of the pathogenicity because such neuraminic acid is also present on cells that are not infected by IBV. It may be that the sialic acid binding is an initial step requiring further contacts to be made perhaps involving a more specific and less generally distributed secondary receptor [3]. Extended and intermittent shedding of IBV from caecal tonsils is evidence for the potential risk of flock to flock transmission via contamination of personnel or equipment. The probability of continuous excretion of the virus at levels usually below the detection limit of the diagnostic tests, or reactivation in sites such as caecal tonsils or kidneys have been already shown [4].

In conclusions, the results of this study clearly corroborate that IRFIBV32 isolate is capable of distribution and proliferation in different tissues with the longest in the intestine of the experimentally infected chicks. It also is a

virulent virus because of its relative long replication period in trachea. The virus titer in the studied tissues had an interesting fluctuation especially in caecal tonsils. Testes and ovaries were the organs that the virus could reactivate without using any chemical. In the last days of the existence in the most tissues the viral titer increased after decreasing and then reached to the zero. It seems that the high titer of the virus is not the only reason for the lesions in the tissues because lesions were present even after disappearing of the virus in the kidneys. In addition, there was no any lesion in the ovaries and testes in spite of the high titer of the virus. More studies are needed to explain more the pathogenesis of the disease.

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