ORIGINAL ARTICLE

Detection of 793/B serotype of infectious bronchitis virus in tissue sample by indirect immunoperoxidase assay

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Abstract The indirect immunoperoxidase (IIP) assay was compared with the reverse transcription–polymerase chain reaction (RT-PCR) for detection of 793/B serotype of infectious bronchitis virus in tissues samples collected from experimentally infected chickens. This technique was optimized in specific pathogen-free (SPF)-embryonated chicken eggs and broiler chickens inoculated with the Iranian IR/773/2001 strain of 793/B serotype The trachea, lung, kidney, and cecal tonsil tissue samples from experimentally infected chicken embryos and chickens were collected in order to prepare tissue sections in IIP assay and to detect in RT-PCR. The sensitivity and specificity values of IIP assay were, respectively, 83 and 84 %, and the positive and negative prediction values were 71 and 91 % when compared with RT-PCR.

Keywords Infectious bronchitis virus · 793/B serotype · Immunoperoxidase assay · RT-PCR

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Introduction

Infectious bronchitis (IB) is a significant viral disease of poultry worldwide and caused by infectious bronchitis virus (IBV). This disease has caused high economic losses in the Iranian poultry industry (Shoushtari et al. 2008; Vasfi-Marandi and Bozorgmehrifard 2001). IBV belongs to group III of the genus *Coronavirus* of Coronaviridae family. The nucleocapsid (N) protein of IBV is closely associated with the genomic RNA, and has highly conserved amino acid and nucleotide sequences. The spike glycoprotein (S) is anchored in the viral envelope and is post-translationally cleaved into two proteins designated S1 and S2. In contrast to the N protein, the S protein is very diverse in terms of both nucleotide sequence and deduced primary protein structure (Kant et al. 1992). The presence of IBV is commonly determined by the inoculation of suspected tissue samples material into embryonated chicken eggs for observation the specific lesions of embryos by the IBVs, followed by serological methods in chickens. IBV also may be detected directly in tissue of infected birds by immunohistochemical (IHC) staining and reverse transcription–polymerase chain reaction (RT-PCR) techniques (Cavanagh and Naqi 2003; De Wit 2000; Swayne et al. 1998). The immunoperoxidase (IP) assay has been successfully used to determine the presence of antigen in tissue sections in most diagnostic laboratories. The IP assay can be performed by direct (DIP) and indirect (IIP) methods. The IIP assay is more sensitive than DIP, but depends on the type selection of reagents. The IIP assay is a reliable and rapid diagnostic assay (Dabbas 2002; Chauhan 1995; Hayat 2002; Levinson and Jawetz 1998). Complexes of antigen and antibody are observable by using enzyme substrate which makes a colorful reaction. The slides prepared according to the IP assay, such as histology and pathology slides, are storable for long time, thus, it is possible to carry out IP assay for retrospective analysis on

paraffin-embedded tissue sections. The main problem of this technique is the existence of endogenous peroxidase in cells such as white blood cells that can show false-positive results. To solve this problem, the activity of endogen peroxidase should be neutralized before using initial antibody.

The aim of this study is optimization of an IIP assay on paraffin-embedded tissue sections prepared from experimentally infected chickens with an Iranian 793/B serotype strain of IBV.

Materials and methods

Virus propagation The Iranian IR/773/2001 (793/B) strain of IBV, isolated at the Razi institute of Iran (Momayez et al. 2008), was used in this study. This virus was propagated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs. The inoculated eggs were incubated at 37 °C for 48–72 h and infected allantoic fluids were harvested 6 days post-inoculation (PI). These fluids were clarified by centrifugation at 8,000×g for 20 min at 4 °C for removing particulate host debris. The supernatant was collected and then the embryo infective dose 50 % (EID_{50}) titer was calculated according to Ried and Mounch's instruction (Swayne et al. 1998).

Experimental infections

Specific pathogen-free-embryonated chicken eggs A volume of 0.1 ml of an Iranian IR/773/2001 (793/B) strain of IBV, H9N2 subtype of avian influenza virus, velogenic Newcastle disease virus, and virulent infectious laryngotracheitis virus (each with approximately of 10^3 EID_{50}), were separately inoculated into the allantoic cavity of five 10-day-old SPF-embryonated chicken eggs. Another five eggs were inoculated with uninfected allantoic fluid of SPF eggs as control group. Inoculated eggs were incubated at 37 °C and checked twice a day. The allantoic fluid and chorioallantoic membrane (CAM) were harvested from eggs at 15, 24, 36 and 48 h after PI. All CAM samples were placed in 10 % formalin buffer solution for 24 h for preparation of paraffin-embedded tissue sections.

Experimental infection in chickens Ten 22-day-old broiler chickens which were negative on ELISA for IBV were selected. Five chickens were inoculated with a volume of 0.2 ml of IBV containing $10^{3.33}$ ELD_{50} titer by oral and tracheal routs. The remaining birds were inoculated with 0.2 ml of uninfected allantoic fluid of SPF embryonated chicken eggs and used as control group. The birds were killed and tracheae, lung, kidney, and cecal tonsil tissues were sampled in days 1, 2, 3, 4, and 5 PI. Each tissue sample was divided to two separate parts. One part was kept at −70 °C for RT-PCR assay, and the other part was put in

10 % formaldehyde buffer solution for 24 h in order to prepare tissue sections for IP assay.

Preparation of paraffin-embedded tissue sections For paraffin-embedded tissue sections preparation, the samples were processed by a passage apparatus. The paraffin blocks were cut by a microtome with a 3–4-μm thickness on the slides covered with 3-aminopropyltriethoxysilane solution. The slides were incubated at 37 °C overnight and used in the IIP assay.

Indirect immunoperoxidase assay The IIP assay was carried out according to the instructions of Naqi (1990) and with slight modifications. The slides were incubated at 56 °C for 30 min. After being rehydrated by ethanol, the slides were rinsed first in deionized water and then placed in Tris buffer solution (TBS=50 mM Tris HCl, 150 mM NaCl, pH=7.6) for 5 min. Antigen retrial was performed by incubating the sections for 20 min with EDTA solution (10 mM Tris, 1 mM EDTA, pH=9). After 20 min, the slides were left at room temperature (RT) and then rinsed with TBS. The tissues were encompassed using a special immunohistochemistry pen. Then, the solutions that block the endogenous peroxidase and biotin activity were added separately on the tissue sections and they were rinsed with TBS after 5 min. A volume of 200 μl of 1/400 dilution of group-specific anti-IBV monoclonal antibody (anti-M and anti-S) (Naqi 1990) was added on tissue sections and incubated at 4 °C for 24 h. Then the slides were rinsed three times in TBS for 5 min. A volume about 200 μl of anti-mouse biotin prepared in goat was added on the slides and incubated at RT for 45 min. After rinsing with TBS as above, the peroxidase conjugated connected to streptavidin was added on the tissue sections and incubated at RT for 15 min. To demonstrate the reaction between antibody and antigen, DAB substrate solution antibody (DakoCytomation Co., Glostrup, Denmark) was used. The slides were removed from the rinsing solution and were dried rapidly. Then, substrate solution was applied on the tissue sections and the sections were placed in RT. After being dehydrated by ethanol, the slides were rinsed first in deionized water then covered with background staining solution (methyl green or hematoxylin). The extra stains were removed by adding ethyl alcohol; finally, the slides were mounted by permanent mounting media (DakoCytomation Co., Glostrup, Denmark). The intensity of reaction of the specific monoclonal antibody against IBV in tissue sections was carried out according to Cruz-Coy et al. (1993). Scoring of reaction was detected by optical microscope in magnification 40 as follows: (0) = no cell is positive, (+1) = less than one positive cell, (+2) = between 1 and 5 positive cells and (+3) = more than 5 positive cells. Furthermore, different dilutions of antigens were analyzed and the 1:300 dilution was taken into account for primary antibody in IIP assay.

Fig. 1 Positive reaction of group-specific monoclonal antibody and infectious bronchitis virus (IBV) on chorioallantoic membrane (*left*) and kidney (*right*) in indirect immunoperoxidase (IIP) assay and DAB is as substrate (×200, ×400)
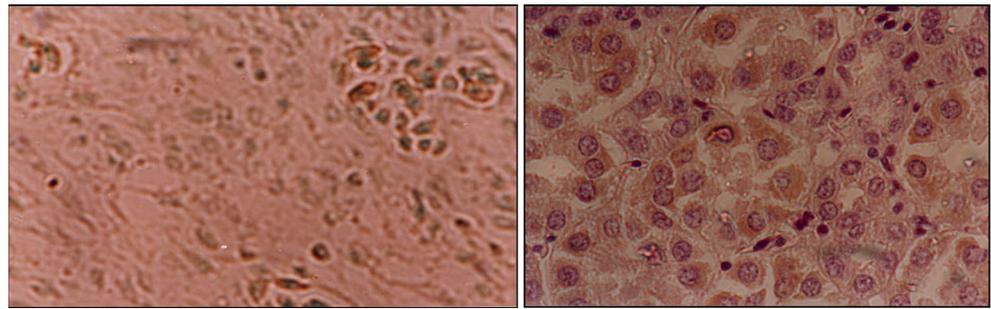
RT-PCR assay

Total RNA extraction Total RNA was extracted from the trachea, kidney, and lung tissues using the Tripure reagent (Life Technologies Inc., USA) according to the manufacturer's instructions. Briefly, 100 mg of the tissues was homogenized with 1 ml of Tripure. After adding 200 µl of chloroform and vigorous shaking the aqueous phase was separated by centrifugation at 12,000×g for 10 min. Total RNA in the supernatant was precipitated by adding 0.5 ml of isopropanol and by centrifugation. After washing and drying, the RNA was dissolved in 15 µl of nuclease-free water.

RT-PCR method Total RNA was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Roche Co., Germany) and (S gene) specific primer (CCAAAGTGCCTTGAGGCCTGG) (AACGCCTGCACCCGTAACGTA) (480 bp) (Titan One Tube RT-PCR System) according to the manufacturer's instructions as described by Cavanagh et al. (1999). Briefly, the amplification was carried out in a 50 µl reaction mixture containing 6 µl of cDNA, 15 pmol of each primer, 200 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$ and 1.5 U of Taq DNA polymerase (Roche Co., Germany) in 35 cycles, each cycle consisting of 95 °C for 1 min, 48 °C for 1.5 min, and 72 °C for 2 min with a final extension at 72 °C for 10 min. The amplified products were separated by electrophoresis, using a 1 % agarose and stained with ethidium bromide. The amplified bands were visualized and photographed under UV illumination (UVP, Transilluminator, M20, USA).

Statistical analysis The kappa statistical method was used in this analysis. The results of RT-PCR were considered as golden standard test. The sensitivity, specificity, and positive and negative prediction values of the experiments have been compared. Furthermore, accuracy and kappa analysis were calculated by evaluating the correlation between experiments according the method described by Dhinakar et al. (1998).

Results

Indirect immunoperoxidase assay The IIP assay of experimentally infected chorioallantoic membrane, tracheae, lung,

Fig. 2 Agarose gel electrophoresis and ethidium bromide staining of RT-PCR products (*M* marker, *ConP* positive control, *T* trachea tissue and 2, 3, 4, and 5 days post-inoculation (PT), *Tcon* trachea tissue negative control, *L* lung tissue, *Lcon* lung tissue negative control, *K* kidney tissue, *Kcon* kidney tissue negative control, *CT* cecal tonsil tissue, *CTcon* cecal tonsil tissue negative control, *ConN* negative control)
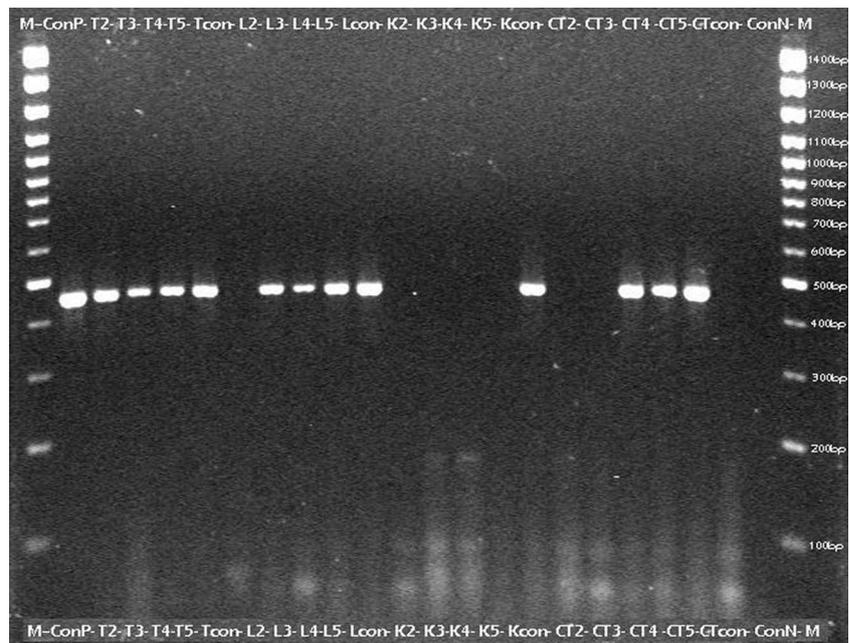
Table 1 Comparison of indirect immunoperoxidase (IIP) and RT-PCR assays in trachea (T), lung (L), kidney (K), and cecal (C) of chickens prepared from experimentally infected (inoculated) and control chickens at 1, 2, 3, 4, and 5 day post-inoculation (PI)

PI	Inoculated chicken								Control chicken							
	IIP				RT-PCR				IIP				RT-PCR			
	Test tissue															
	T	L	K	C	T	L	K	C	T	L	K	C	T	L	K	C
1	−	−	−	−	nd	nd	−	−	−	−	−	−	−	−	−	−
2	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
3	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−
4	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−
5	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−

+ positive reaction, − negative reaction, *nd* not done

kidney, and cecal tonsil tissues was carried out. The IBV-infected cells were brownish orange under the optical microscope examination; whereas no reaction was observed on the tissue sections prepared from inoculation by other heterologous viruses. The 1:300 dilution of the group-specific anti-IBV monoclonal antibody was found as suitable dilution for primary antibody in IIP assay.

Since +1 reaction sometimes was observed in prepared samples in controlled chickens, the intensities of +2 and +3 were considered as positive results in IIP assay. Figure 1 shows the positive response of group-specific monoclonal antibodies of IBV antigens in IIP assay.

Experiment results of RT-PCR Thirty-eight tissue samples of trachea, lung, kidney, and cecal tonsil prepared from experimentally infected chickens were tested by RT-PCR (Fig. 2). The presence of IBV was confirmed in 12 cases, while in 26 samples, it was not confirmed. The presence of virus in days 2, 3, 4, and 5 PI was confirmed in trachea and lung. Furthermore, presence of virus in day 5 in kidney and days 3, 4, and 5 in cecal tonsil were confirmed. However, the existence of IBV in the control tissue samples on days 1, 2, 3, 4, and 5 PI was not confirmed by RT-PCR. The results of RT-PCR and IIP assays in trachea, lung, kidney, and cecal tonsil are shown in Tables 1 and 2.

The IBV was detected in trachea and lung tissues of the inoculated birds on days 3, 4, and 5 in IIP assay; whereas, the positive response was observed in tissue section of kidney on days 2, 3, 4, and 5 in IIP assay. The presence of IBV was confirmed in cecal tonsil tissue sections on days 2, 3, 4 and 5 in IIP assay. The presence of IBV was not detected in tissue samples on days 1, 2, 3, 4, and 5 PI of PBS in IIP assays.

Overall, from total 38 sections of trachea, lung, kidney, and cecal tonsil tissues of ten infected birds tested in RT-PCR only 12 samples (31 %) were positive and 26 samples (68 %) were negative. While 14 (37 %) and 24 (63 %) samples were positive and negative respectively by IIP assay.

Statistical comparison of results Regarding the data, the sensitivity and specificity of IIP assay in comparison with RT-PCR in trachea, lung, kidney, and cecal tonsil are 83 and 84 %, respectively. Furthermore, the positive and negative prediction values of IIP assay were obtained 71 and 91 %, respectively. Accuracy of IIP assay in all tissues was 84 % and kappa coefficient was 94 %.

Discussion

Early diagnosis of poultry diseases in chicken flocks is an important affect in disease control programs. Therefore, rapid diagnostic methods of pathogenic viruses have been developed all over the world. IHC staining techniques have been part of rapid diagnostic techniques that have been developed much more and have been used increasingly for rapid diagnosis of poultry viral diseases (Swayne et al. 1998).

The use of these techniques in retrospective studies have been applied and available for analysis of diseases in one area by using frozen or paraffin-embedded tissue sections

Table 2 Detection of infectious bronchitis virus (IBV) by indirect immunoperoxidase (IIP) and RT-PCR assays in trachea, lung, kidney, and cecal tissues of inoculated and control chickens at 1, 2, 3, 4, and 5 day post-inoculation (PI) (a: tissue with positive reaction and b: total tissues)

PI	Inoculated chicken		Control chicken	
	IIP	RT-PCR	IIP	RT-PCR
1	0[a]/4[b]	0/2	0/4	0/4
2	2/4	2/4	0/4	0/4
3	4/4	3/4	0/4	0/4
4	4/4	3/4	0/4	0/4
5	4/4	4/4	0/4	0/4
Total (percent)	14/20	12/18	0/20	0/20
	(70 %)	(66 %)	(0 %)	(0 %)

(Fitzgerald et al. 1994). Some researchers have used these techniques in diagnosis of other poultry viral diseases, such as *Birnavirus*, *Paramyxovirus*, and *Orthomyxoviruses* (Guy et al. 1997; Liu et al. 2000; Lockaby et al. 1993; McNulty et al. 1990; Shamsaddini-Bafti et al. 2002; Smyth et al. 1993; Swayne et al. 1996).

Bhattacharjee et al. (1994); Owen et al. (1991); De Wit (2000); Chen et al. (1996), and Nakamura et al. (1991) have used IHC techniques for rapid diagnosis of IBV antigens. Owen et al. (1991) diagnosed the presence of IBV with indirect immunofluorescence and IIP assays in the mucous and under mucous of trachea, kidney, and cecal tonsil. They observed the presence of virus in the histocyte and lymphocyte cells.

In this study, IIP assay was used on paraffin-embedded tissue sections for diagnosis of IBV, and the competency of these techniques was assisted by RT-PCR assay. For routine usage of IHC techniques, these methods should be standardized. Therefore, an experimental infection was carried out in 6-week-old chickens. Besides, for getting suitable dilution of antibodies, an experimental infection was carried out in SPF chicken eggs. Determination of these dilutions was repeated in the chickens under the experiments. The IIP and RT-PCR assays were carried out on trachea, lung, kidney, and cecal tonsil tissues on the days 1, 2, 3, 4, and 5 PI (Tables 1 and 2). There was high correlation between the results of RT-PCR and IIP assays.

Naqi (1990) used IP assay for diagnosis of IBV, identifying the virus within 15 h PI in chorioallantoic membrane. Swayne et al. (1998) used IP assay with other diagnostic assays for assessment of IBV pathogenesis. They reported that IP assay is an acceptable, sensitive, reliable and economic technique that is simultaneously capable of diagnosis of the presence of antigens on tissue samples and analysis of cells constructions.

Handberg et al. (1999) reported that the competency of IBV diagnosis is 82 % with RT-PCR in inoculated chickens. While this value was nearly 60 % in the IIP assay. Chen et al. (1996) detected the IBV antigens in the kidney tissue after 4 days with IIP assay.

In this study, sensitivity, specificity, and positive and negative prediction value of IIP assay by using monoclonal antibodies against conserved section of S and M of IBV on paraffin-embedded tissue section from experimentally infected samples are 83, 84, 71, and 91 %, respectively. In addition, the accuracy of this experiment with RT-PCR was 96 % that shows complete conformity. Regarding reaction intensity, monoclonal antibodies anti-S and M of IBV were used for increasing sensitivity and specificity of IIP assay for IBV antigens on tissue sections. Using IHC techniques, it is recommended for detecting of IBV antigens in the tissues especially in trachea and lung tissues. Particularly, IHC techniques are recommended for laboratories that have no facility for virus separation but equipped with histopathology techniques.

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