

Experimental study on histopathological changes and tissue tropism of Iranian infectious bronchitis serotype 793/B-like virus in SPF chickens

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Avian infectious bronchitis virus (IBV) is prevalent in all countries with intensive poultry flocks. This disease is characterised primarily by respiratory signs, but some IBV strains may also infect other organs such as the intestinal and urogenital tracts. The aim of this study was to characterise the histopathological lesions and tissue tropism of Iranian isolate IR/773/2001(793/B) of avian infectious bronchitis virus in different organs of experimentally infected SPF chickens. Forty-two one-day-old, specific pathogen-free (SPF) chicks were divided randomly into two groups (21 chicks to each group). At the age of 12 days, one group was inoculated intra-ocularly with 10^3 EID₅₀ of the 793/B isolate, and the other was kept as the control group. Tissue samples were collected at 2, 4, 6, 8, 10 and 12 days post-inoculation (PI). The IBV virus was detected in the caecal tonsils and cloaca from the 2nd to the 12th day PI. The virus was also detected in the kidneys from days 4–10 PI and in the bursa of Fabricius from days 4–12 PI. The virus was detected in the trachea, lungs and thymus. The most obvious histopathological lesions were found in the trachea, kidney, lungs and bursa of Fabricius. Amongst the lymphoid tissues, histopathological changes were found most frequently in the bursa of Fabricius. The results of this study indicated that the 793/B serotype of IBV is unlikely to cause mortality, severe clinical signs or gross lesions in infected chickens, but its replication in some tissues including the bursa of Fabricius could render birds susceptible to other micro-organisms.

Introduction

Infectious bronchitis (IB) is an acute, highly contagious and economically important viral disease that occurs in commercial chickens of all ages. It is caused by the infectious bronchitis virus (IBV) (Cavanagh & Gelb 2008), a member of the genus *Gammacoronavirus*, family *Coronaviridae*, with more than 26 serotypes (Enjuanes *et al.* 2000; King *et al.* 2012). Infectious bronchitis was first reported in the USA in 1931 as a respiratory disease (Schalk & Hawn 1931). Some strains of IBV also infect non-respiratory tissues including reproductive tissues (Farzinpour, Nili & Hosseini 2009; Van Roekel *et al.* 1951), kidneys (Cumming 1962, 1963; Winterfield & Hitchner 1962), and the alimentary tract (Yu *et al.* 2001).

Whilst the 793/B serotype was first identified in Britain in 1990–1991, it was subsequently confirmed that the virus had been present in France since 1985 (Gough *et al.* 1992; Parsons *et al.* 1992; Picault *et al.* 1995). The first isolation of IBV in Iranian chicken flocks was reported in 1994 (Aghakhan *et al.* 1994). Later, several Iranian researchers identified the 793/B serotype. (Vasfi Marandi, Bozorgmehrifard & Karimi 2000; Momayez *et al.* 2002; Seify abad Shapouri *et al.* 2002). This serotype turned out to be one of the predominant types of IBV circulating in Iran (Nouri, Assasi & Seyfi-Abad Shapouri 2003; Shoushtari *et al.* 2008). However, this serotype was not further studied and therefore its pathogenic properties and tissue tropism are not well characterised.

The aim of the present study was to investigate the pathogenic traits of 793/B serotype in SPF chickens as well as to determine tissue distribution and histopathological changes in various organs. Clinical signs and gross and microscopic lesions were evaluated and viral nucleic acid was assessed in several tissues of challenged chicks by reverse transcription polymerase chain reaction (RT-PCR) and nested PCR.

Materials and methods

Virus: The Iranian isolate IR/773/2001 (793/B serotype) of IBV used in this study was isolated in Iran (Momayez *et al.* 2002) and classified by RT-PCR, nested-PCR and sequencing (Toroghi *et al.* 2004). The titre of the virus was determined by inoculation of 0.1 ml of each 10-

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fold serial dilution (10^{-3} – 10^{-9}) with phosphate-buffered saline (PBS) of the virus stocks into the chorioallantoic cavity of 10-day-old SPF embryonated eggs. The titres were expressed as the 50% embryo-infective dose (EID50) calculated by the method of Spearman-Karber (Gelb & Jackwood 1998; Villegas 1998).

Chickens: Forty-two white Leghorn chicks hatched from specific pathogen-free (SPF) embryonated chicken eggs (Venky's, India) were divided randomly into two groups (21 chicks per group). They were kept separately in isolators under positive pressure at the Razi Vaccine and Serum Research Institute, Karaj-Iran. All the chicks were provided with feed and water *ad libitum*.

Experiments: At the age of 12 days, all birds in the experimental group were inoculated with chorioallantoic fluid containing 10^3 EID50/0.1 ml IBV serotype-793/B by eye drop. The other group was kept as the control group. After challenge, all the chickens were monitored daily for clinical signs and mortality. On days 2, 4, 6, 8, 10, 12 post inoculation (PI), three chickens from each group were randomly selected and used for sample collection. They were then humanely euthanised, necropsies were performed and gross lesions were recorded. Tissue samples, which included trachea, thymus, lungs, spleen, kidneys, caecal tonsil, bursa of Fabricius, and cloaca, were aseptically collected for virus detection using the RT-PCR technique and to evaluate histopathological changes.

Histopathology: Tissue sections were fixed for 24 h – 48 h in 10% neutral buffered formalin. The tissues were routinely processed and embedded in paraffin blocks, sectioned at 5 µm, deparaffinised and stained with haematoxylin and eosin (H&E).

RNA extraction: All tissue samples were homogenised with tryptose phosphate buffer, centrifuged for 5 min and the supernatant liquid was stored at -70°C until use. RNA was extracted from the samples using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions.

RT-PCR: RT-PCR was performed using the Titan one-tube RT-PCR system (Roche Applied Science, Mannheim, Germany). For the amplification reaction, two primers common to all types of IBVs, XCE1+ and XCE2- (Table 1) (Adzhar *et al.* 1997), both from the S1 gene, were used with 4 µl of the extracted RNA in a final volume of 50 µL. For the RT reaction, the mixture was incubated at 45°C for 45 min and then heated to 94°C for 2 min. The PCR reaction was performed using an Eppendorf thermal cycler (Mastercycler gradient) for 35 cycles of denaturation (94°C , 1 min), annealing (48°C , 1 min), extension (68°C , 1 min), followed by a final extension (68°C , 10 min).

Nested PCR: The nested PCR was performed with oligonucleotide XCE3- common to all three strains and the oligonucleotide BCE1+ (Table 1) specific for type 793/B

(Adzhar *et al.* 1997). In the case of the positive samples, a nested PCR was performed with 1/10 dilution of the first reaction in a final volume of 25 µl but the negative samples were not diluted. The nested-PCR reaction consisted of 25 cycles of denaturation (94°C , 1 min), annealing (48°C , 1 min), extension (72°C , 1 min) and a final extension (72°C , 10 min). The final products were analysed by electrophoresis in a 1% agarose gel, stained with SYBR safe (Invitrogen) and visualised by UV transillumination (UVP).

Results

Clinical findings

Some chickens of the infected group showed mild gasping and depression at 2 days PI. The signs were less severe after 4 days PI. There was no mortality in any of the groups during the experiment. Feed consumption and weight gain were reduced in the infected group in comparison with the control group. There were no clinical signs in the control group.

Gross necropsy findings

There were no detectable gross lesions in any of the organs of the control chickens. The chickens exposed to IBV showed the following lesions: in the tracheas, slight congestion and serous exudates were seen at days 2 and 4 PI; thereafter the tracheas were macroscopically normal. The kidneys were pale and swollen from day 4 to day 10 PI; thereafter the kidneys gradually returned to normal. The thymus and bursa of Fabricius were shrunken at days 4 and 6 PI, respectively.

None of the birds in either group died during the experiment and there were no clinical signs or gross lesions in the control group.

Histopathology

There were no detectable lesions in any of the organs collected from the control chickens. In contrast, the chickens exposed to IBV manifested the following lesions: the tracheas revealed deciliation, epithelial and glandular desquamation, and slight congestion and oedema were seen in the tracheas on day 2 and day 4 PI. Proliferation of undifferentiated epithelium was initially seen at day 2 PI but mucous gland differentiation was seen from day 4 PI (Figure 1). By day 6 PI, there was tracheitis, with congestion, oedema and hyperplasia of the epithelial layer (Figure 2). Infiltration of lymphocytes and a

TABLE 1: Reverse transcription polymerase chain reaction and nested-polymerase chain reaction primer sequences and positions of the oligonucleotide.

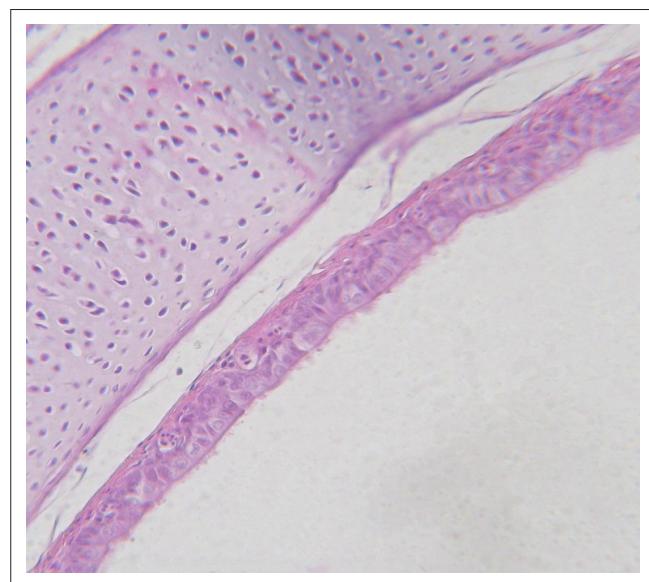
| Oligonucleotide | Sequence (5'-3') | Gene | Location | Size of amplicon |
|-----------------|----------------------|------|----------------------|------------------|
| XCE1+a | CACTGGTAATTTCAGATGG | S1 | 728–749 ^c | 464 bp |
| XCE2-b | CTCTATAAACACCCCTACA | S1 | 1168–1193 | |
| BCE1+ | AGTAGTTTGTGTATAAACCA | S1 | 958–978 | 154 bp |
| XCE3- | CAGATTGCTTACAACCACC | S1 | 1093–1111 | |

Source: Table adapted from Adzhar *et al.*, 1997's article Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain Published in *Avian Pathology*
a, Positive sense oligonucleotides; b, negative sense oligonucleotides; c, the numbers correspond to the nucleotide positions in the indicated references; bp, base pairs.

few heterophils were also seen at day 6 PI in the tracheas. At day 8 PI, the severity of the lesions decreased and by day 12 PI the regeneration was complete.

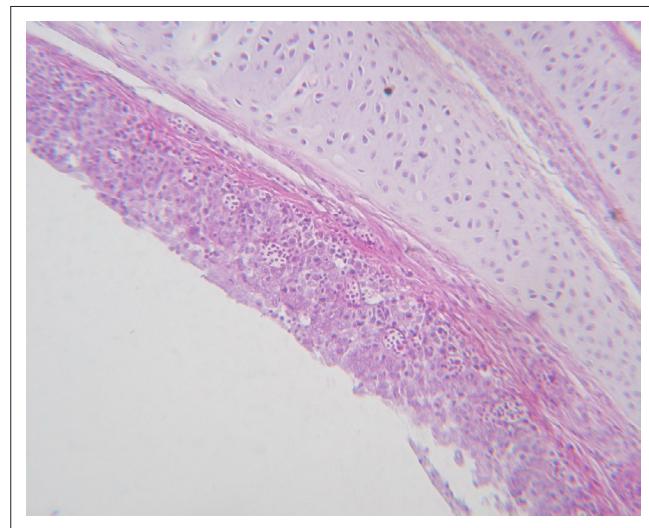
In the kidneys, congestion, haemorrhage and multifocal necrosis of the renal tubules were seen from day 2 to day 8 PI. Focal infiltration of lymphocytes was seen between the urinary ducts. By the day 6 PI, cystic tubules containing cellular casts and foci of mononuclear leukocyte infiltration were prominent, mostly in the medulla. The first sign of regeneration of the tubular epithelium was seen at day 8 PI and by day 10 PI most of the cell debris was cleared from the lumina of the tubules (Figure 3).

The most prominent histopathological changes in the lungs were seen at days 2–6 PI, with congestion, haemorrhage, oedema and infiltration of lymphocytes into the submucosa



Haematoxylin and eosin stain $\times 120$.

FIGURE 1: Trachea – desquamation and deciliation of epithelial cells, 2 days post-inoculation.



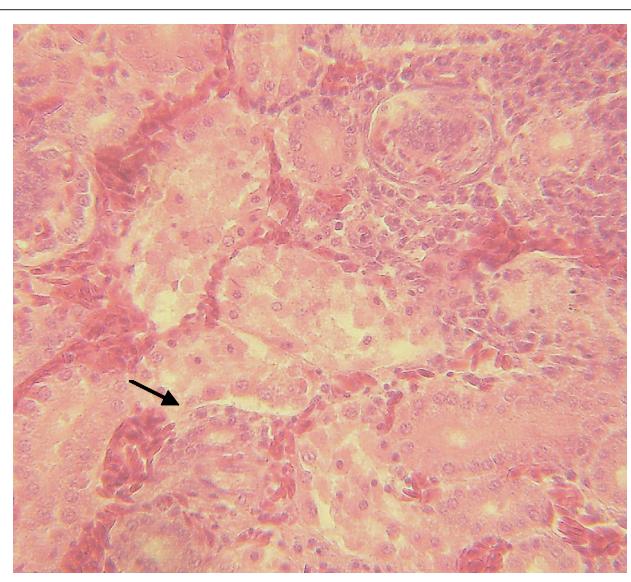
Haematoxylin and eosin stain $\times 170$.

FIGURE 2: Hyperplasia of the epithelial layer cells and infiltration of lymphocytes and heterophils in the trachea, 6 days post-inoculation.

of secondary bronchi. Congestion and haemorrhage of the parabronchi were seen at day 2 and day 4 PI, and fibrin exudates and focal infiltration of lymphocytes were observed at days 4 and 6 PI. Regeneration of the epithelial cells began from 6 days PI (Figure 4).

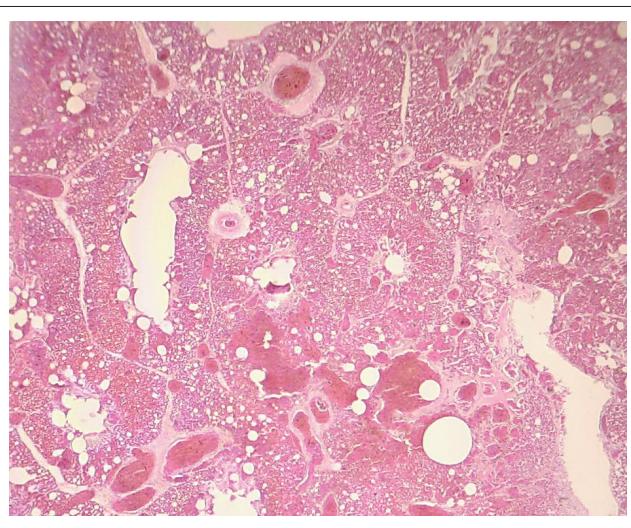
The bursa of Fabricius had the highest frequency of histopathological changes affecting lymphoid tissue. Mild depletion of some follicles and cyst formation in the epithelial layer were observed at day 2 and day 4 PI (Figure 5). However, at day 6 PI, signs of acute inflammation with necrosis and infiltration of heterophils were seen. By day 8 PI, folding of the epithelial layer and fibroplasia was seen.

The most prominent histopathological change in the thymus was seen at day 6 PI, consisting of thinning of the cortical



Haematoxylin and eosin stain $\times 430$.

FIGURE 3: Congestion, haemorrhage and focal necrosis of urinary tubules (arrow) and lymphocyte infiltration in the kidney, 10 days post-inoculation.



Haematoxylin and eosin stain $\times 150$.

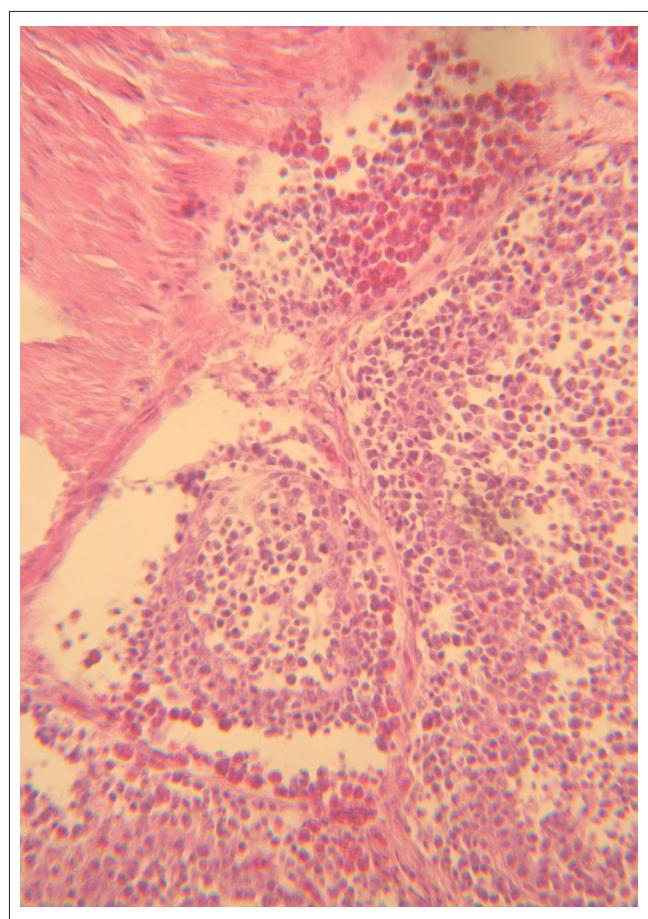
FIGURE 4: Congestion and haemorrhage in lung tissue at 4 days post-inoculation.

regions and a few necrotic foci in the central region, which was infiltrated with heterophils.

Mild to moderate depletion of white pulp with reticuloendothelial cell hyperplasia was seen in the spleen at days 2–6, but the most severe changes were at day 6 PI. Lymphoid hyperplasia was observed from 8 days PI.

Virus detection in tissues following virus inoculation

The presence of the virus was evaluated in all the samples taken during the experiment from both inoculated and control chickens. The virus was not detected in samples taken before inoculation in the experimental group or those taken from the control group. It was detected in all samples except the spleen taken from the experimental group at



Haematoxylin and eosin stain $\times 430$.

FIGURE 5: Acute inflammation accompanied by necrosis and heterophil infiltration in the bursa of Fabricius, 6 days post-inoculation.

TABLE 2: Virus detection in various tissues of chicken after inoculation with 793/B serotype of infectious bronchitis virus.

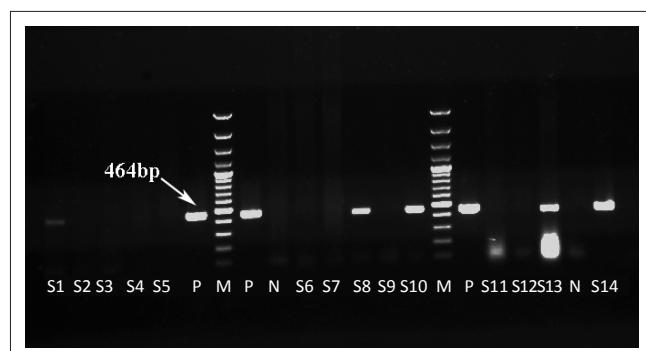
| Days PI | Trachea | Lungs | Caecal tonsils | Kidneys | Spleen | Bursa of Fabricius | Thymus | Cloaca |
|---------|---------|-------|----------------|---------|--------|--------------------|--------|--------|
| 2 | + | - | + | - | - | - | - | + |
| 4 | + | + | + | + | - | + | - | + |
| 6 | - | - | + | + | - | + | + | + |
| 8 | - | - | + | + | - | + | + | + |
| 10 | - | - | + | + | - | + | - | + |
| 12 | - | - | + | - | - | + | - | + |

+, positive samples; -, negative samples.
PI, post-inoculation.

different times PI. The typical results of the RT-PCR test for virus detection (Figure 6) and of the nested PCR test (Figure 7) were visualised by UVP. Furthermore, the distribution of the virus in different tissues following inoculation was examined (Table 2).

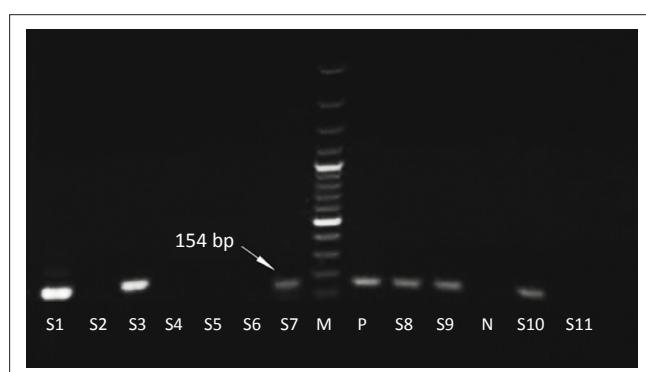
Discussion

The first outbreak of IBV in Iranian chicken flocks was reported in 1994 (Aghakhan *et al.* 1994). Outbreaks of 793/B serotype were subsequently reported by several researchers in Iran (Vasfi Marandi *et al.* 2000; Momayez *et al.* 2002; Seify abad Shapouri *et al.* 2002) and since then, IB has been recognised as an economically important disease in the Iranian poultry industry. Although IBV pathogenesis has been studied by various techniques (Hofstad & Yoder



M, Marker 100 bp; N, Negative control; P, Positive control.
S1 and S8, trachea; S2 and S9, lungs; S3 and S10, caecal tonsils; S4 and S11, kidney; S5 and S12, spleen; S13, bursa of Fabricius; S6, thymus; S7 and S14, cloaca.

FIGURE 6: Detection of infectious bronchitis virus by Reverse transcription polymerase chain reaction in different tissue samples.



M, DNA marker 100 bp; P, Positive control; N, Negative control; bp, base pairs.
S1, caecal tonsil; S2 and S11, spleen; S6 and S7, lungs; S4 and S10, thymus; S5 and S9, trachea; S3 and S8, bursa of Fabricius.

FIGURE 7: Detection of 793/B serotype by nested polymerase chain reaction in different tissue samples.

1966; Crinion & Hofstad 1972; Gough *et al.* 1992; Chong & Apostolov 1982; Mahdavi *et al.* 2007a,b; Benyeda *et al.* 2009, 2010), the present study was conducted to determine the pathogenesis, tissue tropism and pathological changes due to a 793/B-like strain of IBV reported in Iranian poultry flocks (Momayez *et al.* 2002).

Parsons *et al.* (1992) and Capua *et al.* (1994) noted that the replication of IBV in the respiratory tissues causes characteristic but not pathognomonic signs such as gasping, coughing, tracheal rale and nasal discharge. Occasionally, puffy, inflamed eyes and swollen sinuses may be seen (Parsons *et al.* 1992; Capua *et al.* 1994). McMartin (1993) reported that the upper respiratory tract is the primary site of IBV replication, after which a viraemia occurs and the virus spreads widely to other tissues. Results of a study using seven strains of IBV demonstrated that birds inoculated with 793/B serotype had no clinical signs or mortality (Benyeda *et al.* 2010). In uncomplicated cases, food consumption and weight gain were significantly reduced within 3 days after infection and the chickens became depressed (Otsuki, Huggins & Cook 1990). In this study, the only clinical signs were depression and reduced weight gain compared with the control group, which is in agreement with previous reports (Benyeda *et al.* 2009, 2010; Capua *et al.* 1994; Dhinakar & Jones 1996; Mahdavi *et al.* 2007a; Momayez *et al.* 2002; Otsuki *et al.* 1990; Parsons *et al.* 1992).

Mahdavi *et al.* (2007b) detected viral antigen in the tracheal epithelial cells within 2 day to 5 day PI. Benyeda *et al.* (2010) detected 793/B antigen in the trachea 4 days PI, whilst no antigen was found at 7 days PI. In another study, the 793/B serotype virus was detected in one-day-old SPF chicks 7 days PI, but in six-week-old broilers the virus was detected 3 days PI (Picault *et al.* 1995). Some researchers have detected IBV in the trachea for longer periods (Ambali & Jones 1990; Otsuki *et al.* 1990; Boroomand, Asasi & Mohammadi 2012). In this study, the 793/B serotype virus was detected in the trachea only at 2 and 4 days PI. It seems that this serotype of virus could replicate for long periods in the trachea in the presence of the other pathogens, or in the first days of life. In the present study, similar histopathological changes were observed in the trachea to those reported by Nakamura *et al.* (1991), and Purcell and McFerran (1972), which included three stages, namely degeneration, hyperplasia and recovery.

Some strains of IBV are nephropathogenic and replicate in respiratory tissues and kidney, but the lesions are more evident in the kidney (Albassam, Winterfield & Thacker 1986; Butcher, Winterfield & Shapiro 1989; Ignjatovic *et al.* 2002; Kinde *et al.* 1991; Lee *et al.* 2004; Purcell, Tham & Surman 1976). Several reports confirmed that 793/B-like strains replicate in the kidneys and produce lesions in them (Albassam, Winterfield & Thacker 1986; Dhinakar & Jones 1996; Benyeda *et al.* 2009, 2010; Boroomand *et al.* 2012). The tubular epithelial cells of the kidney are the target cells of IBV (Owen *et al.* 1991; Janse, van Roozelaar & Koch 1994).

IBV was detected in the kidneys in various studies, up to 7 days PI (Dhinakar & Jones 1996; Benyeda *et al.* 2009). Several studies have demonstrated that the 793/B-like strains induce milder histopathological lesions in the kidneys in comparison with the QX-like strains (Benyeda *et al.* 2009, Boroomand *et al.* 2012). On the other hand, it was reported that renal lesions due to infection with a 793/B-like isolate of virus consisted of tubular degeneration, desquamation and necrosis of the epithelium and an inflammatory cell reaction in the interstitium (Mahdavi *et al.* 2007a) and it was suggested that the virus has a tropism for the epithelium, especially that of the collecting tubules of the kidney (Mahdavi *et al.* 2007b). Histopathological changes developed from 2 days PI and were seen in all parts of the kidney and the lesions were constant during this experiment. These changes included congestion, haemorrhage, necrosis of urinary ducts and lymphocytic infiltration. The virus was also detected as early as 2 days PI to 10 days PI. These results were consistent with previous reports about this serotype of IBV (Benyeda *et al.* 2009, 2010; Mahdavi *et al.* 2007a,b; Momayez *et al.* 2002; Owen *et al.* 1991).

Benyeda *et al.* (2009) reported mild pathological signs and limited virus replication in respiratory tissue caused by the isolate of 793/B serotype and showed that this isolate was classified as being of low pathogenicity because it caused mild histopathological lesions. In day-old chicks infected with Italy 02 IBV serotype, viral nucleic acid was only detected in the lungs 9 days PI by *in situ* hybridisation. In addition, no positive staining was observed in lung tissue (Dolz *et al.* 2011). Viral antigen has been detected in the epithelial cells of the lungs (Janse, van Roozelaar & Koch 1994). On the other hand, Benyeda *et al.* (2010) detected viral antigen in the lung of infected chicks with some QX-like isolates, but they could not detect antigen in chicks infected with 793/B and M41 strains, and they noted that there was no significant difference between the lesions in the lungs produced by various strains. Furthermore, viral antigen was detected in the epithelium and in alveolar mucous glands as early as 2 days PI (Mahdavi *et al.* 2007b). Our results revealed the presence of the virus only at days 4 PI by RT-PCR. Histological changes were evident on day 4 and day 6 PI, after which recovery was observed in lung tissue.

Nucleic acid of the virus was detected in epithelial cells of the nasal turbinates, trachea, lung, kidney, caecal tonsil, cloaca and bursa of Fabricius of day-old chicks by *in situ* hybridisation, and massive replication in enterocytes of the caecal tonsil and rectum was also reported (Dolz *et al.* 2011). Scattered positive cells were observed in the epithelium of the bursa of Fabricius from 3 to 7 days PI. Scattered positive enterocytes were observed in the rectum from 18 to 27 days PI and in the caecal tonsil up to day 24 PI (Dolz *et al.* 2011). The consecutive results from immunohistochemistry show that the virus first replicates in the trachea and lungs and then the intestinal and especially the renal epithelial cells become infected with the virus (Chong & Apostolov 1982; Janse, van Roozelaar & Koch 1994; Owen *et al.* 1991). Results of a study with the 793/B-like virus revealed that the virus

persisted longer in the trachea and lung in SPF chicks than in broilers but virus detection in the Harderian gland and bursa was similar (up to 7 days PI); virus clearance from the kidney in both types of birds appeared to be completed by day 10 PI (Raj & Jones 1996).

Isolation of IBV from the bursa after experimental H52 and H120 infections has been described previously (Ambali & Jones 1990; MacDonald & McMartin 1976). Nucleic acid was also detected in epithelial cells of the trachea, lung, intestine, and bursa of embryos inoculated with eight strains of IBV 2 days PI (Lee, Brown & Jackwood 2002).

It was recently reported that the 793/B-like serotype is more enterotropic than pneumotropic and is also associated with diarrhoea in broilers (Boroomand *et al.* 2012; cf. Dhinakar & Jones 1996). Although IBV has a tropism for gut tissues, no gross or histological changes have been reported. The results of this study revealed that the virus was detected in the caecal tonsils and cloaca during the whole study period, which is in accordance with previous reports about virus replication in gut tissues.

Although isolation of IBV from the spleen has been described previously (Otsuki *et al.* 1990), in this study, the virus was not detected in the spleen. However, reticular tissue hyperplasia and lymphatic depletion in this organ were observed histologically.

Conclusion

The results of this study indicated that the 793/B-like serotype of IBV did not cause mortality, severe clinical signs or gross lesions in experimentally infected SPF chickens, but its replication in the bursa of Fabricius and gut tissues and the lesions induced could expose the birds to other diseases. Further studies are required to confirm the role of the strain and of co-infection with other pathogens like avian influenza virus, *Escherichia coli* and *Ornithobacterium rhinotracheale* (ORT) on the outcome of this disease.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

P.B. (Science and Research Branch, Islamic Azad University) performed the experiments, including preparing the samples and doing the examinations and wrote the manuscript. R.M. (Razi Vaccine and Serum Research Institute) was the project leader. M.H.H. (Razi Vaccine and Serum Research Institute) performed all the histopathological examinations. M.H.B.

(Science and Research Branch, Islamic Azad University) and S.A.P. (Razi Vaccine and Serum Research Institute) assisted in the experimental and project design.

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