

The infectious bronchitis vaccine strain virus is more pathogenic in chicken embryos than the wild virus strain 2575/98

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TSAI, CHENG-TA, HUE-YING CHIOU, CHING-HO WANG: The infectious bronchitis vaccine strain virus is more pathogenic in chicken embryos than the wild virus strain 2575/98. Vet. arhiv 86, 699-709, 2016.

ABSTRACT

An avian infectious bronchitis virus (IBV) strain 2575/98 was attenuated using serial chicken embryo passage to become a vaccine in Taiwan. The aim of this study was to investigate the replication ability, pathogenicity, and tissue tropism of the wild and vaccine strains in chicken embryos. The embryos were inoculated with different titers of wild and vaccine strains. Quantification of virus in allantoic fluid was evaluated using real time RT-PCR. The results showed that the vaccine strain replicated in higher titers than the wild strain, and caused embryo death so quickly that only a few dwarfisms occurred. The embryos inoculated with wild and vaccine strains had similar lesions that were confined primarily to the chorionallantoic membrane (CAM), liver, and kidneys. The immunohistochemical data showed that IBV was present predominantly in the lungs, kidneys, and CAM. Although both strains caused hepatic damage, very few virus antigens were detected in the hepatic tissue. The pathogenicity of the vaccine becomes higher in embryos although it is lower in chickens than its wild strain. The vaccine strain could be used as a possible new vaccine candidate for IBV control.

Key words: immunohistochemistry, infectious bronchitis, pathogenicity, vaccine

Introduction

Avian infectious bronchitis (IB) caused by the IB virus (IBV) is an acute, highly contagious infectious disease in chickens around the world. This illness causes great economic loss in the poultry industry (JACKWOOD and DE WIT, 2013). It belongs to the family *Coronaviridae*, genus *Gamacoronavirus*, containing spike (S), membrane and nucleocapsid proteins. In spite of vaccination, IB has occurred from time to time due to little cross protection from different virus serotypes or genotypes (HUANG et al., 2005).

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Extensive diversity and different types appear due to its rapid replication, high mutation rate, and genome recombination (JACKWOOD and DE WIT, 2013; ABDEL-MONEIM et al., 2009).

As a result of many IBV serotypes, different multiple live-attenuated IB vaccines are in use around the world (HUANG et al., 2006a). IBV vaccines are attenuated using multiple serial passages, in embryonated eggs (HUANG et al., 2006b). As the IBV is passaged, the virus gradually adapts to SPF chicken embryos. Consequently, SPF chicken embryo lesions become more and more apparent, and the time to death period is gradually shortened (FENG et al., 2015; JACKWOOD and DE WIT, 2013).

It is well known that the avian influenza virus (SCHOLTISSEK et al., 1988) and Newcastle disease virus become less virulent after attenuation in chicken embryos (ALEXANDER and JONES, 2008), whose pathogenicities are parallel to both embryos and chickens. Thus, their pathogenicity to chickens could be determined in chicken embryos. Indeed, the virulent Newcastle disease virus has a mean death time in embryos of less than 60 hours after embryo inoculation (ALEXANDER and JONES, 2008). In contrast, the IBV vaccine strain which adapts to chicken embryos shows higher pathogenicity in embryos but less pathogenicity in chickens (HUANG et al., 2006a). The purpose of this study was to compare the biological characteristics of the wild and vaccine strains of IBV in chicken embryos.

Materials and methods

Viruses. Avian infectious bronchitis virus, strain 2575/98 (TW-I) (briefly 2575), was passaged in SPF chicken embryos 77 times to obtain a vaccine strain (HUANG et al., 2006b). Its fourth passage in specific-pathogen-free (SPF) chicken embryos (Animal Health Research Institute, Chidin, Taiwan) was used as the wild strain and the 77th passage as the vaccine strain in this study.

The virus titers for embryo inoculation were determined by the presence of death or dwarfism in chicken embryos, and expressed as EID₅₀. All inoculated embryos were sacrificed at 7 days post-inoculation (DPI) (16 days old) if not dead. The pathogenicity of IBV in chicken embryos was evaluated by the death rates and death times, post-inoculation. The virus concentrations in the allantoic fluid from virus-inoculated embryos were quantified using real-time RT-PCR.

Quantify IBV in allantoic fluid of embryos by real-time RT-PCR. Fifty-four (54) 9-day-old SPF chicken embryos were divided into two groups, with 27 embryos each. The groups of embryos were inoculated with 10 EID₅₀ of wild and vaccine strains, respectively. The inoculated embryos were observed daily and the allantoic fluid was collected from inoculated embryos at 1, 2 and 3 DPI.

The virus quantities of IBV in embryos were determined using real-time RT-PCR with specific primer for the IBV S1 gene. The sequences were rC2U: 5'TGGTT GGCA(T/C) TTACA (A/C/T)GG(A/G/T)3', rC3L: 5'(A/G)CAAT GTGTA ACAAA (T/C)ACT3' (HUANG and WANG, 2007). RNA samples were reverse-transcribed for 120 min at 37 °C with a High Capacity cDNA Reverse Transcription Kit, according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA).

Quantitative PCR was performed under the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 1 min at 60 °C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 nM of forward and reverse primers. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicate. The exact copy concentration of the target gene was determined by absolute quantification, relating the CT value to a standard curve.

Construction of standard curves. The PCR product from primers specific for the S1 gene was amplified by PCR using rC2U/rC3L primer. The PCR product was cloned into a yT&A vector (Yeastern Biotech, Taipei, Taiwan). The concentration of the cloned plasmid was measured using a spectrophotometer. A 10-fold serial dilution series of the cloned plasmid, ranging from 1×10^5 to 1×10^9 copies/ μ L, was used to construct the standard curve. The cycle threshold values in each dilution were measured in triplicate, using real-time PCR to generate the standard curve.

Pathogenicity in chicken embryos. Serial ten-fold dilutions of the virus, $10^{1.7}$, $10^{2.7}$, $10^{3.7}$, $10^{4.7}$ egg infectious dose 50 (EID_{50}), were inoculated into five to ten 9-day-old SPF chicken embryos. The embryos were kept at 37 °C for 7 days and examined daily. Those that died after inoculation were examined for their gross and microscopic lesions. All embryos were sacrificed 7 DPI (16 days old) for pathological examination and IBV gene quantification with real-time RT-PCR.

Five chicken embryos without inoculation were used as the control in each experiment. The dwarfing embryos were confirmed by the embryo sizes after IBV inoculation. The sizes of the live IBV-inoculated embryos were compared with the age-matched non-inoculated control embryos. Dwarfism was defined as a chicken embryo weighing less than 75% of the mean for the un-inoculated embryos of the same age. However, embryos before 13 days old (4 DPI) were not examined for the presence of dwarfism because their sizes were too small to evaluate.

Dwarfing effect of wild and vaccine strains in embryos inoculated with 1 EID_{50} . Both wild and vaccine strains were adjusted to 1 EID_{50} and inoculated into 26 9-day-old SPF chicken embryos, respectively. The lethal and dwarfing effects of both strains on chicken embryos were examined daily and terminated at 7 DPI.

Pathology. All embryos that died or were killed at 7 DPI were necropsied and examined for the presence of gross lesions in the lungs, liver, kidneys, and chorionallantoic membrane (CAM), which were then fixed in 10% neutral-buffered formalin. Tissues were routinely processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for microscopic examination. Five chicken embryos without inoculation were used as the control in each experiment.

Immunohistochemical (IHC) staining. Immunohistochemical (IHC) staining was performed using the advanced Super Sensitive™ Polymer-HRP IHC Detection System (BioGenex Lab., Fremont, CA), according to the manufacturer's instructions, with a mouse anti-IBV monoclonal antibody, QI3-4a (CHEN et al., 2011). Tissue sections at 5 µm were laid on coated slides, air-dried at room temperature, deparaffinized, rehydrated successively in 100%, 90%, 80%, and 70% ethanol for one min each, and then washed in deionized water for 5 min. After treatment with 100 µg/mL proteinase K (AppliChem Inc, St. Louis, MO) for 15 min at room temperature, the tissue slides were gently washed in tris-buffered saline Tween-20 (TBST) buffer for 5 min, and then incubated in 3% H₂O₂ for 10 min to quench the activity of endogenous peroxidase. After rinsing with deionized water, the tissue slides were gently washed in TBST buffer for 5 min and then incubated with the PowerBlock reagent at room temperature for 10 min. Following rinsing with deionized water, the tissue slides were gently washed in TBST buffer for 5 min and then incubated with QI3-4a antibody at a 500X dilution at room temperature for 60 min. After washing in TBST buffer, the tissue slides were then incubated with the SuperEnhancer (BioGenex HRP kit, Fremont, CA) at room temperature for 30 min, washed in TBST buffer for 5 min, and drained. The tissue slides were incubated with the Polymer-HRP reagent at room temperature for 40 min, rinsed with TBST buffer, and drained. Following treatment with the chromogen-DAB at room temperature for 3 min, the slides were rinsed with TBST buffer, drained, and counterstained with Mayer's hematoxylin for 1 min. After rinsing with deionized water, the slides were covered with a coverslip using aqueous mounting media. Positive and negative controls were run in parallel in each assay. The positive control was an IHC staining-positive lung tissue block from an IBV case. Negative controls included corresponding tissue sections from IB virus-negative tissues, and substitution of the monoclonal antibody with phosphate buffered saline (PBS). Samples were considered positive for IB virus only when distinct red-brown, finely clumped granular cytoplasmic deposits were revealed in the samples and positive control, but no non-specific false-positive signals in the negative controls.

Statistical analysis. The differences in death rates caused by wild and vaccine strains were compared using the chi-square test or Fisher's exact test. The virus copy numbers using real-time RT-PCR were expressed as mean ± SD and compared using the Student t test. The significance level was set at P≤0.05.

Results

Quantification of viruses in embryos. The virus genome copy numbers in the chicken embryos inoculated with wild and vaccine strains at 10 EID₅₀, respectively were determined using real-time RT-PCR. The copy numbers were higher in chicken embryos inoculated with the vaccine strain than those inoculated with wild strain (Table 1). The results showed that the vaccine strain replicated more quickly and higher than the wild strain in chicken embryos. It was worth to noting that the allantoic fluid showed high virus titers on 1 - 2 DPI. IBV titers declined sharply later on.

Table 1. The IBV quantification of wild and vaccine strains in chicken embryos inoculated with 10 EID₅₀

Strain	1 DPI ^A	2 DPI	3 DPI
Wild	54.1 ± 30.3 (9) ^B	26.7 ± 31.6 (12)	15.4 ± 15.5 (6)
Vaccine	60.7 ± 72.8 (9)	70.7 ± 61.5 (12)*	52.8 ± 34.7 (6)*

A: DPI means days post inoculation; B: mean ± SD (n), genome copy number (10⁸); *: significant difference between wild and vaccine strains (P<0.05).

Pathogenicity and dwarfing effects in chicken embryos. The pathogenicity of IBV in the chicken embryos was evaluated by death rates and death times post-inoculation with different virus titers. The death rates of the embryos inoculated with the wild strain were titer-dependent (Table 2). The higher titers of the wild strain caused higher embryo death. Only 10% of the inoculated embryos died at the early stage. In contrast, the IBV vaccine strain caused embryo death mostly at the early stage (2-4 DPI), even reaching 50% at a low titer of 10^{1.7} EID₅₀. In general, the vaccine strain caused earlier and higher death rates in chicken embryos than the wild strain (P<0.05).

Table 2. Comparison of embryo death rates and times after inoculation with different titers of wild and vaccine strains

Strain	Virus titer (10 ⁿ EID ₅₀)	Death number after inoculation		Total death rate (%) ^B
		2-4 DPI ^A	5-7 DPI	
Wild	4.7	1	2	3/5 (60)
	3.7	0	2	2/5 (40)
	2.7	1	2	3/10 (30)
	1.7	0	1	1/10 (10)
Vaccine	4.7	5	0	5/5 (100)
	3.7	4	1	5/5 (100)
	2.7	10	0	10/10 (100)
	1.7	5	1	6/10 (60)

A: DPI means days post inoculation; B: The total death rates of the vaccine strain are significantly higher than those of the wild strain (P<0.05).

The dwarfing embryos weighed less than 75% of the normal embryo weight at the corresponding ages and were curled into a spherical form, a typical lesion from IB infection. The early death embryos were excluded from the dwarfing effect count since they died at the early stage after inoculation, during which the embryo sizes were too small to recognize dwarfism.

The vaccine strain caused embryo death so quickly that only a few stunting instances occurred (Table 3). Conversely, the wild strain caused less embryo death and more dwarfism ($P < 0.05$). About one half (6/13) of the embryos escaped killing from the 1 EID₅₀ vaccine strain and showed dwarfing in the later stage (Table 3).

Table 3. The death and dwarfing effect from wild and vaccine strains on chicken embryos inoculated with 1 EID₅₀

Strain	Inoculated number	Early death (2-4 DPI) ^A	Dwarfism	Total infection
Wild	26	1	12	13
Vaccine	26	7	6	13

A: DPI means days post inoculation

Pathologic lesions in virus-inoculated embryos. Both the wild and vaccine strains caused similar lesions in chicken embryos. Tissues from dead embryos showed more obvious lesions than those that were still alive at same time, including white spots in the liver, urate deposition in the kidneys, and hemorrhage in embryos or CAM. Besides insufficient embryo weight, the amnion was thickened and adhered closely to the embryo. The dwarfing embryo was curled into a spherical form with its deformed feet over its head.

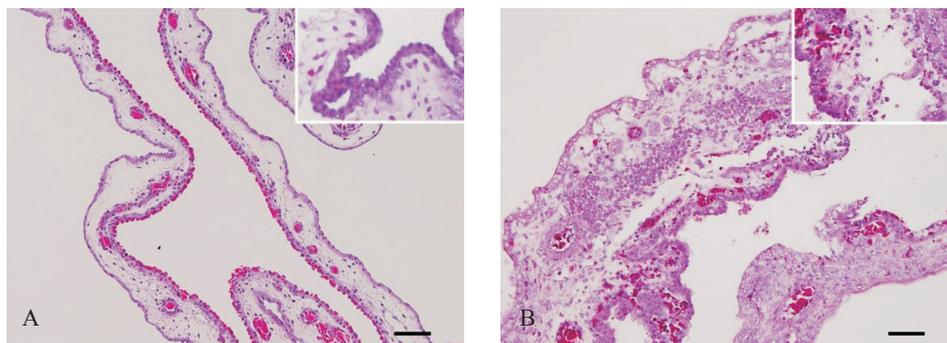


Fig. 1. A) Normal chorioallantoic membrane from 15-day-old embryo without inoculation. B) Chorioallantoic membrane from 13-day-old embryo inoculated with the wild IBV strain. The chorionic epithelial and allantoic epithelial cells are destructed. The mesoderm layer is full of eosinophilic cells and debris. (H&E stain, $\times 200$, scale bar = 50 μm . Insert: $\times 400$).

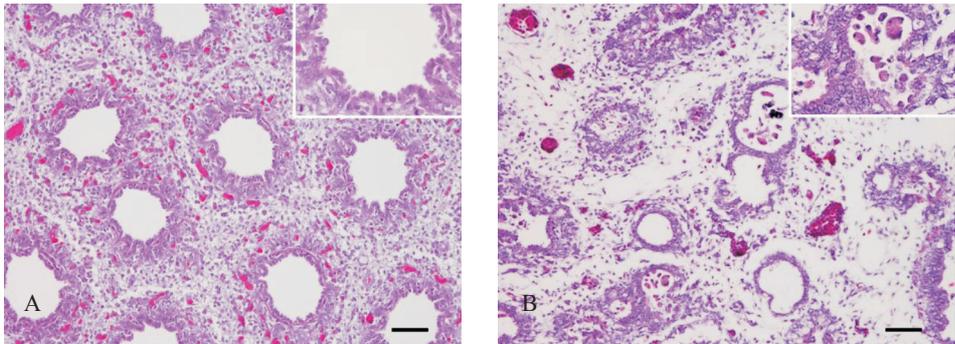


Fig. 2. A) Normal lung from a 15-day-old embryo without inoculation. B) lung from a 16-day-old embryo inoculated with the vaccine strain. Air capillary shows inflammation and edema. Cell debris is found in parabronchial lumen. (H&E stain, $\times 200$, scale bar = 50 μm . Insert: $\times 400$).

Histopathological changes were found in the CAM, lung, liver, and kidneys in embryos inoculated with wild and vaccine strains. Both strains induced almost the same lesions. The chorionic epithelial and allantoic epithelial cells were destroyed. The mesoderm layer was full of eosinophilic fluid and cell debris (Fig. 1). The lungs showed severe edema, epithelium damage of the parabronchus, filled with cell debris (Fig. 2). Increased numbers of hematopoietic centers around the portal vein and focal necrosis without inflammatory cells were found in the livers (Fig. 3).

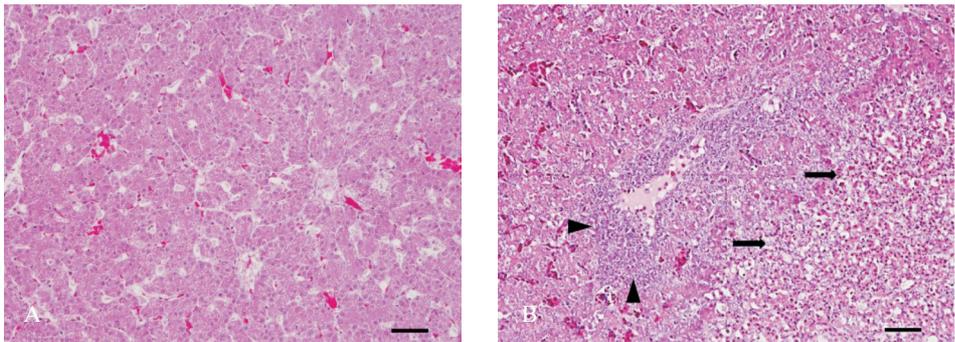


Fig. 3. A) Normal liver from a 15-day-old embryo without inoculation. B) Liver from a 16-day-old embryo inoculated with the wild strain. Focal and massive necrosis (arrows) with a few inflammatory cells and hematopoietic centers (triangles) are noted in the hepatic tissue. Although necrosis is present in liver, very few IBV antigen was found in the entire hepatic tissue by Immunohistochemical staining. (H&E stain, $\times 200$, scale bar = 50 μm).

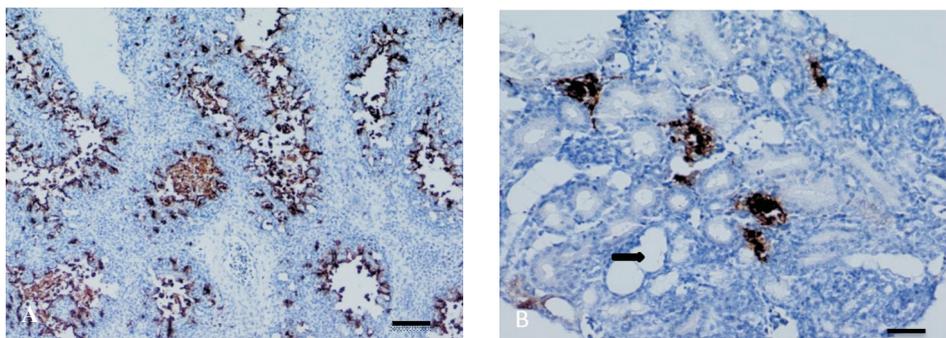


Fig. 4. Immunohistochemical staining with anti-IBV monoclonal antibody, QI3-4a. A) Lung from a 16-day-old embryo inoculated with the vaccine strain. The parabrachial lumens are full of virus antigen in cell debris. B) Kidney from a 16-day old embryo inoculated with the vaccine strain. A few collecting tubules show massive virus antigen. On the contrary, some dilated collecting tubules caused by urate contain no virus antigen (arrow). ($\times 200$, scale bar = 50 μm)

The IHC results revealed that the target sites of both strains were parabrachial epithelial cells (Fig. 4A), collecting tubules of the kidneys (Fig. 4B) and the serosa of the spleen. IHC results showed that virus antigen was found but only a few in the splenic parenchyma, and very few in the hepatic parenchyma. Although showing severe necrosis, the hepatic tissue contained very few virus antigens.

Discussion

Contrary to other viruses, such as Newcastle disease virus and avian influenza virus, our results showed that the IBV vaccine strain is more pathogenic in chicken embryos than its original wild strain. This result is consistent with BIJLENGA's report (2004) that the embryos were still alive after virus inoculation at lower passage in chicken embryos from a wild IBV strain but died from an embryo-adapted IBV strain after higher embryo passages. Furthermore, the dwarfism of chicken embryos was unrelated to the death rates of chicken embryos, but did relate to the death times after inoculation. When the virus titers reduced, some chicken embryos lived for more than 4 DPI and they showed dwarfism. Therefore, dwarfism became more prominent when the chicken embryo lived longer. Bijlenga reported that the dwarfing effect of embryos caused by the adapted virus disappeared at higher passages (BIJLENGA et al., 2004). However, in our study, the dwarfism phenomenon reappeared when we decreased vaccine virus titers to inoculate chicken embryos (data not shown). The wild strain induced higher levels of dwarfism than the vaccine strain, because the latter caused embryo death within 2-4 DPI (Table 2). That time is too short for the embryos to show dwarfism.

As a vaccine, the 2575 vaccine strain must replicate in the embryos to a high titer. The present study showed that the vaccine strain replicated much better than the wild strain ($P < 0.05$). However, both strains grew rapidly in the early days. They reached their maximum level in one to two days post inoculation and then decreased sharply after four DPIs. The growth kinetic curves of these IBVs showed the same growth kinetics as stated in previous reports (ABDEL-MONEIN et al., 2009; DARBYSHIRE et al., 1975).

As the IBV strain 2575 was passaged, the virus increasingly adapted to the chicken embryos (HUANG et al., 2006b). Consequently, the time to death gradually shortened. This result is in agreement with results reported in the same conditions with other IBV strains (FENG et al., 2015). Embryo death occurs as early as 2-3 DPIs and with increasing passage in embryos (JACKWOOD and DE WIT, 2013). However, some embryos escaped killing from low doses of vaccine IBV and showed dwarfism (Table 3). Dwarfism indicated that the embryo is still alive when compared to age-matched embryos without inoculation. The reason why the wild strain induces higher levels of dwarfism than the vaccine strain is that the latter causes embryo death. The time is too short for the embryos to show obvious dwarfism.

Although the livers showed parenchymal damage, very few IBV antigens were found in the IHC. The hepatic lesions were therefore not caused by IBV itself. They might have been caused by hypoxia or excessive urate or other ions, which needs further investigation (WANG, 2014). It is evident that the embryo kidney and lung cells are much more sensitive than liver cells (LUKERT, 1965). Detecting IBV antigens using IHC is a good method to investigate the presence of viral antigens in tissue. The presence of IBV antigens in inoculated eggs, found using an antigen detection method, is independent from the occurrence of embryo lesions (JACKWOOD and DE WIT, 2013). The hepatic necrosis was not directly caused by the virus. Necrosis might be caused by the indirect effect of IBVs, such as ionic hypoxia (GERRITZEN et al., 2006; WANG, 2014).

Although the vaccine strain caused early mortality, its virus titer was enough to be used as a vaccine (HUANG and WANG, 2006b). The 77th passage showed less pathogenicity in chickens and preserved its immunogenicity (HUANG et al., 2005). This study shows that this 2575 vaccine strain replicates well in chicken embryos, and could be a good candidate for vaccine production. In conclusion, for this IB strain 2575, the vaccine possesses higher pathogenicity in chicken embryos than its original wild strain.

Acknowledgements

The financial support from the Council of Agriculture, Taiwan, is highly appreciated.

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TSAI, CHENG-TA, HUE-YING CHIOU, CHING-HO WANG: Cijepni soj virusa zaraznog bronhitisa patogeniji je za pileće zametke od divljeg soja 2575/98. Vet. arhiv 86, 699-709, 2016.

SAŽETAK

Virus zaraznog bronhitisa peradi soj 2575/98 bio je oslabljen uzastopnim pasažama u pilećim zametcima da bi poslužio kao cijepni soj u Tajvanu. Cilj je ovog rada bio istražiti mogućnost umnožavanja, patogenost i tropizam terenskog i cijepnog soja u pilećim zametcima. Zametci su bili inokulirani cijepnim sojem različitog titra. Količina virusa u alantoisnoj tekućini bila je određena RT-PCR-om u stvarnom vremenu. Rezultati su pokazali da se cijepni soj umnažao u višem titru od divljeg soja i prouzročio uginuće zametaka tako brzo da se uspelo razviti svega nekoliko kržljavih. U inokuliranih zametaka, bez obzira na divlji ili cijepni soj, razvile su se slične promjene pretežito na korioalantoisnoj opni, jetrima i bubrezima. Imunohistokemijski je dokazano da se virus zaraznog bronhitisa prvenstveno nalazio u plućima, bubrezima i korioalantoisnoj opni. Iako su oba soja prouzročila oštećenja jetara, neznatna količina virusnog antigena bila je dokazana u jetrenom tkivu. Patogenost cijepnog soja bila je u zametcima jača, a u pilićima slabija od divljeg soja. Cijepni soj bi se mogao rabiti kao mogući kandidat za proizvodnju novog cjepiva protiv zaraznog bronhitisa.

Ključne riječi: imunohistokemija, zarazni bronhitis, patogenost, cjepivo
