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Identification of a Nephropathogenic Strain of Infectious Bronchitis Virus (IBV) in Some Northern Provinces in Vietnam

[1]Tran Duc Hoan, [1]Doan Thi Thao, [1]Nguyen Dinh Nguyen, [2]Wang Bin and [3]Nguyen Luan

[1]Faculty of Animal Science and Veterinary Medicine, Bacgiang Agriculture and Forestry University, Bichdong Street, Vietyen District, Bac Biang Province, Vietnam
[2]Clinical Veterinary Medicine Department, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China
[3]Faculty of Veterinary Medicine and Fishery, Fisheries and Technical Economic College, Dinhbang Street, Tuson City, Bacninh Province, Vietnam

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Corresponding Author:
Tran Duc Hoan
Faculty of Animal Science and Veterinary Medicine, Bacgiang Agriculture and Forestry University, Bichdong Street, Vietyen District, Bac Biang Province, Vietnam
Email: dr.hoan288@gmail.com

Abstract: The nine-day-old Specific Pathogen-Free (SPF) chicken embryos were inoculated with the XDC-2 strain of Infectious Bronchitis Virus (IBV). 50% Embryo Infectious Dose (EID_{50}) and pathological lesions were detected in the developing embryo. At the same time, 18-day-old SPF chickens were inoculated with the same strain of IBV in case of 5 chickens were injected with sterile PBS (1X, pH 7.4) as control and the chickens were checked daily for the symptoms. At 14 days' post-challenge, all the chicks were slaughtered, the pathological examinations and the virus was detected by RT-PCR. The results indicated that the virus caused dwarfism and death of the embryo after 3 days of post-challenge. The EID_{50} of the virus was estimated to be $5\times10^{-5.33}$/mL. All the experimental chickens showed the symptoms after 3 days of infection such as depression, ruffled feathers, and slight diarrhea. The dead chickens were examined for gross lesions, which showed kidney enlargement, nephropathy, and massive uric acid salt deposits. Histopathological examination revealed that the kidney had tubular ectasia, glassy degeneration of epithelial cells, endothelial cell necrosis and exfoliation in some vessel lumens, monocyte infiltration, hyperemia, and hemorrhage in the renal interstitium. The virus was isolated and detected in the kidney by RT-PCR. It indicated the IBV XDC-2 was a predominantly nephrotoxic infectious bronchitis virus.

Keywords: Infectious Bronchitis Virus, Identification, Nephritogenic

Introduction

Avian Infectious Bronchitis Virus (IBV) causes an acute, highly contagious viral disease and high mortality, particularly in chickens less than 6 weeks old. However, based on its clinical manifestations, the disease can be separated into respiratory, genital, digestive, and nephropathogenic types. Mostly the respiratory form of IBV is described as commonly associated with other respiratory diseases in the chickens. The respiratory type of IBV may affect the kidneys and in laying flocks it decreases egg yield and quality (Cavanagh, 2007). Since the beginning of the 20th century, a lot of research has been reported in China and worldwide about the prevalence of nephrotoxic IBV isolates (Ji *et al.*, 2020; Liu *et al.*, 2006; Yu *et al.*, 2001). All studies revealed that vaccine strains were grouped in different clusters with the major types of neuropathogenic infectious bronchitis viruses circulating in China (Liu *et al.*, 2006). The IBV vaccine strains at present in China were correlative mainly with the Massachusetts, such as H120 and Ma5, and the Connecticut strains, but the recent research was found differently from the Massachusetts or Connecticut. So, this is the reason why IB has occurred frequently in vaccinated flocks in China (Chen *et al.*, 2021; Liu *et al.*, 2009).

The IBV genome is approximately 27 kb in length (Boursnell *et al.*, 1987; Sutou *et al.*, 1988) and codes for four main structural proteins including Spike glycoprotein (S), a small Envelope protein (E), Membrane glycoprotein (M) and Nucleocapsid protein (N) (Spaan *et al.*, 1988; Sutou *et al.*, 1988). The S glycoprotein comprises two glycopeptides: S1 and S2. Neutralizing and serotype-specific antibodies have mainly directed against the S1 glycoprotein. As the IBV has a single-stranded RNA genome point mutations or genetic recombination may lead to the new variants which could cause immune failure. IBV evolution is currently considered to be driven

by three factors such as the inherent property of its RNA genome to mutate. These three factors probably role in the performance and involve various mechanisms, such as point mutations, deletion, insertions, and recombination to generate new variants (Li *et al.*, 1994; Yuan *et al.*, 2023). More than 20 serotypes within IBV as defined by virus neutralization have been identified worldwide (Gough *et al.*, 1992; Yu *et al.*, 2001). Initially, it was revealed all the isolates belonged to a single prototype termed the Massachusetts (Mass) serotype isolated from commercial poultry (Cavanagh and Naqi, 2003). Subsequently, other serotypes were isolated and it is now clear that a considerable number of different serotypes with antigenic and pathogenic differences exist in different parts of the world (Gough *et al.*, 1992).

Natural outbreaks of IBV are often the result of infections with vaccine strains that differed serologically (Ali *et al.*, 2022; Feng-long *et al.*, 2004; Wang *et al.*, 1997). It was observed that the small differences in S1 contributed to cross-protection poorly (Cavanagh *et al.*, 1997; Li *et al.*, 1994). Thus, the molecular identification of IBV has been focused on the analysis of the S1 protein gene. Initial molecular characterization of the Chinese isolates of IBV resulted in the identification of a new genotype as compared to the S1 genes with those of IBVs in America, European countries, and Japan and IBV isolates in Taiwan and China (Liu and Kong, 2004; Ma *et al.*, 2019). Isolation and identification of new IBV variants and their genotyping (S1 gene sequence homology analysis) are necessary to design the strategy for the prevention and control of disease (Cavanagh, 2007; Xu *et al.*, 2007). In this study, the nephropic strain of IBV (XDC-2) was identified and its pathogenicity was evaluated in 10-day-old SPF chicken embryos and experimental chickens. This study may provide some interesting data that might be beneficial to improve the efficacy of the vaccines for IBV infection in poultry production.

Materials and Methods

Animal Ethics Approval

All animal experiments in this study were conducted according to guidelines for laboratory animal use and were legally approved by the institutional animal care and use committee of the Vietnam veterinary association.

Collection of Samples

We collected 300 sick chicks of different ages suffering from severe clinical signs of infectious bronchitis disease in four provinces (Bacgiang, Bacninh, Hanoi, and Thainguyen) of northern Vietnam from September 2019 to August 2021. All sick chicks were slaughtered to collect the internal organs to prepare for RNA extraction and PCR detection.

Embryonated Eggs and Chickens

The Specific Pathogen-Free (SPF) chicken embryos and the live chickens were purchased from the hatchery at Vang Village, Bichdong Street, Vietyen District, Bac Giang Province, Vietnam. A crumbled chick starter diet was provided *ad libitum* during the course of the experiments. The use of all laboratory animals and animal subjects was approved by the institutional animal care and use committee of the Vietnam Veterinary Association.

Virus Isolation and Propagation

The following method was used for the inoculation of IBV in the allantoic cavity of a ten-day embryo. Briefly, the ends of the egg were inoculated and the eggshell punch was swabbed with a cotton wool soaked in 70% alcohol and allowed to evaporate. Mortality between 2-7 days' post-inoculation was considered to be virus-specific. The allantoic fluids were harvested as follows; the eggs were chilled at 4°C for at least 2 h to kill the embryo and to reduce the contamination of the allantoic fluid with blood during harvesting. Removed the wax and each egg was swabbed with cotton wool soaked with 70% alcohol to disinfect and remove condensation from the shells. The fluid was collected into sterile containers and stored at -80°C for further experiments. The harvested fluid was tested for the absence of bacteria and fungi. Dead embryos were examined for the presence of embryo stunting, curling, urate in the mesonephros, or focal necrosis in the liver. After 3rd day post inoculation, five live embryos were also removed from the incubator and were placed at 4°C for 24 h and the chorioallantois fluid of the embryos was collected for the next passage. Harvested fluid was made in five blind passages in chicken embryos prior to being considered negative for IBV isolation.

Virus Titration

The blood samples were collected from chicken veins. After collection of the blood into an anticoagulant (3.8% solution of sodium citrate), the cells were washed in phosphate-buffered saline (PBS 1X, pH 7.4) and transferred to a container suitable for centrifugation. The PBS was added to fill the container. Mixed it gently and centrifuged at 1500 rpm for 10 min. With the help of a Pasteur pipette or a 10 mL glass pipette, the supernatant was removed. The pellet of red blood cells was not disturbed. The washing steps were repeated twice.

Strains and isolates of IBV agglutinate chicken Red Blood Cells (RBCs) after neuraminidase treatment (Ruano *et al.*, 2000; Schultze *et al.*, 1992). For this purpose, the IBV was treated with the final conc. Of 5 percent trypsin and incubated at 37 for half an hour. The isolated IBV was firstly treated with 50 µL of virus added to each well containing 50 µL of normal saline (0.9% NaCl) two-fold serial dilutions were made and

virus titers were measured by Hem Agglutination (HA) assay as described elsewhere (G. R. S, 1997). Briefly, the HA assay was performed in V-shaped 96-well plates. Two-fold serial dilutions of virus samples were mixed with an equal volume of a 0.5% suspension of washed chicken RBCs and incubated at room temperature for 20-30 min. Wells containing a suspended, homogeneous layer of RBCs were scored as positive, and bead formation was scored as negative.

Determination of 50 Percent Embryo Infectious Dose (EID_{50})

The endpoint of the titration is used to calculate the infectivity titer of the original suspension of the virus. The 50% chicken Infection Dose (EID_{50}) was determined by inoculating serial 10-fold dilutions of the virus into SPF embrocated chicken eggs via the allantoic cavity route of inoculation and the titers were expressed as 50% (median) Embryo Infectious Doses (EID_{50}). 10-fold serial dilutions were used for titrations and at each dilution, 4 embryos received 0.2 mL inoculum. The embryos were candled daily and examined for 1 week for showing the characteristic IBV lesions such as dwarfing, stunting, or curling of embryos, were recorded as infected by IBV.

The Reed and Muench formula to calculate the index is:

$$\text{Index} = \frac{(\%\text{ infected at dilution immediately above 50\%})}{(\%\text{ infected at dilution immediately above 50\%}) - (\%\text{ infected at dilution immediately below 50\%})}$$

Experimental Infection of Chicken

18 SPF chickens were divided at random into 3 groups and were housed separately. XDC-2 strain was used for experimental infection on the 18th day by oculonasal application. Groups 1 and 2 were inoculated with 10^3 EID_{50} allantoic fluid 1 mL/each and group 3 was mock inoculated with PBS (1X, pH 7.4) and served as the control. The chicks were examined daily for signs of infection up to 14 days after inoculation. Directly after sampling, some of the kidney and lung tissues were stored individually in a formaldehyde medium and some were stored at -20°C until the virus was identified.

Histopathological Analysis

For histopathological analysis, first of all, tissues were cut into small pieces of about 0.5-1cm (width and length) and fixed in 10% buffered formalin (formalin 1: 9 phosphate buffer saline) for 24 h. After the next 24 h formalin was changed to wash out any blood cells or cell debris and new buffered formalin was added. After 24 h, tissue samples were put in the processing chambers and the lid of the chamber was closed by cutting the excess part of the tissue. Tissue samples were marked accordingly to identify the type of tissue and infected or control groups. Tissues were sliced into 5-µm sizes by Microtome and stained with hematoxylin and eosin stain. Pathologic changes were observed under an Olympus microscope (Axio Lab A1, Carl Zeiss, Germany).

Virus Recovery by RT-PCR

Tissues that were taken at the same time point in each group post-inoculation were pooled for virus isolation. The pooled samples containing 10.000 U penicillin and 10.000 µg streptomycin were inoculated into 4 SPF embryos via the allantoic cavity (0.2 mL per egg). The eggs were candled daily and the allantoic fluids of the inoculated embryos were collected 4 days post-inoculation for RT-PCR amplification and characteristic IBV lesions such as dwarfing, stunting, or curling of embryos.

Viral RNA was extracted from 200 µL of infectious allantoic fluid by TRIzol reagents following the manufacturer's protocol. RT was performed with M-MLV Reverse transcriptase using the reverse primer P2. The RT procedures were performed using 7 µL of RNA in a 20 µL reaction volume. Owing to genetic variation among IB strains, it is difficult to use one set of Polymerase Chain Reaction (PCR) primers amplifying the S1 gene of all IBV strains. The degenerate set of primers was constructed on the basis of related sequences of the S1 gene of nephropathogenic IBV isolates from China submitted to the Gene bank in NCBI. The sequences were analyzed using MEGA version 5 software. PCR was carried out using a degenerate set of primers as follows:

- P1: 5' ATGTTGGTGAAGTYAMTDTT 3'
- P2: 5' AMACAAGRTCACCATTTA 3'

The PCR program was described as follows. The amplification was performed in a 50 µL reaction mixture containing 1.5 mM $MgCl_2$, 1× PCR buffer, 0.2 mM of dNTP, 20 pM of each forward and reverse primer, 1.5 U of Taq DNA polymerase (Promega, Madison, USA) and 2 µL of cDNA. The PCR reaction was run in a thermocycler (PTC-150) with the following program: Initial denaturation at 94°C for 5 min, then 30 cycles as Denaturation at 94°C for 40 sec; Annealing at 58°C for 45 sec; Extension at 72°C for 1-2, 2 min and the final extension at 72°C for 10 min was performed. Then the PCR products were analyzed on 1.0% agarose gel.

Results

Determination of Chicken Embryos Inoculated with EID_{50}

The four dilutions of virus from 10^{-2}-10^{-6} were inoculated separately in SPF chicken embryo at 0.2 mL per egg and incubated for 4 days at 37°C. The result showed that the development of embryos was significantly less than the normal embryo, while some appeared curled and feathered,

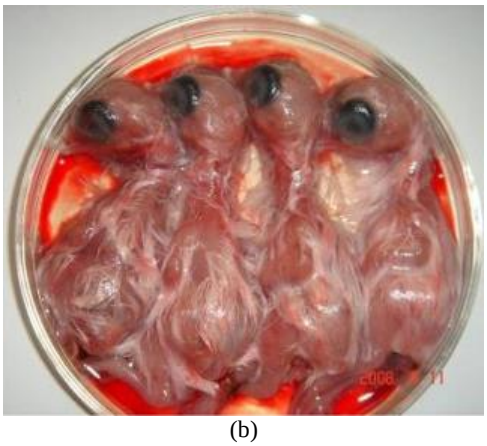
with dysplasia, often bleeding in the skin, congestion, dead and dwarf embryo (Fig. 1). EID_{50} value of $5\times10^{-5.33}$/mL was calculated (Table 1).

Clinical Signs and Gross Lesions

The incidence of virus attack chicken was 100%. After 5 dpi the chicken started to show the clinical signs suggestive of IBV which were mild respiratory symptoms, decreased appetite, increased water intake, depression, reduced neck, feathers messy, drooping; have mild pasty diarrhea. Kidney swelling, tubular dilatation, and deposition of uric acid salt. 12 dpi majorities of the chickens were morbid and mortality was also at peak. Death status after induced infection of the XDC-2 strain of IBV showed mortality from 16.7-33.3% with virus dilution at the 10^3 EID_{50} (Table 2).

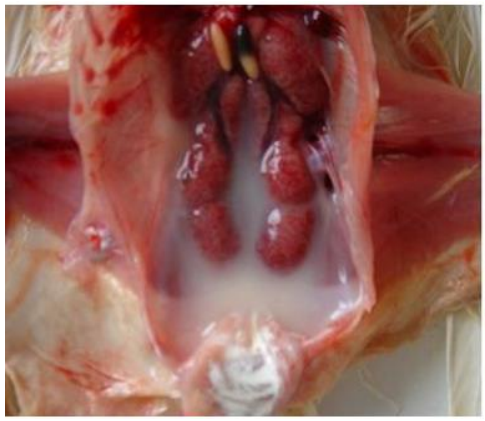
The necropsy of the dead chickens revealed a small amount of light-yellow viscous liquid within the trachea, kidney enlargement, showing the typical spotted kidney; tubal thickness, and a lot of urate deposition (Fig. 2). No abnormal changes in other organs were noted.

(a)

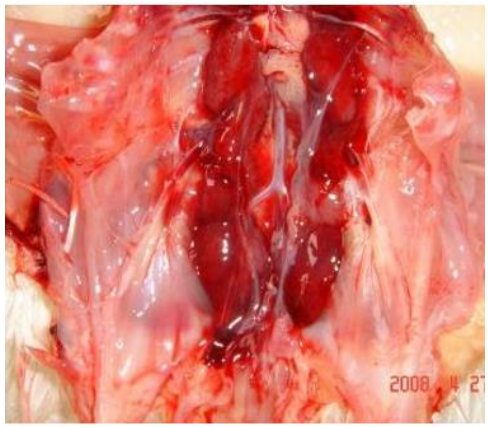


(b)

Fig. 1: Effects of the XDC-2 strain of IBV on embryonic development after 5 days post-inoculation



(a)



(b)

Fig. 2: Pathological changes after induced infection of XDC-2 strain after 5 days post-inoculation; (a) Dead chicken showed kidney enlargement, spotted kidney, tubular thickness, and urates deposition; (b) Chicken from the control group showed normal kidneys

Histopathological Changes

The kidneys of the IBV inoculated and PBS (1X, pH 7.4) inoculated groups were removed both from the dead and morbid chicken after 14 dpi. After staining with H and E stain, these were compared. The IBV inoculated group showed tubular dilatation, hyaline degeneration of epithelial cells, most of the glass cylinder lumen, and necrosis of epithelial cells, which can be seen in the interstitial mononuclear cell infiltration (Fig. 3). The control group has no abnormality.

PCR Identification of IBV

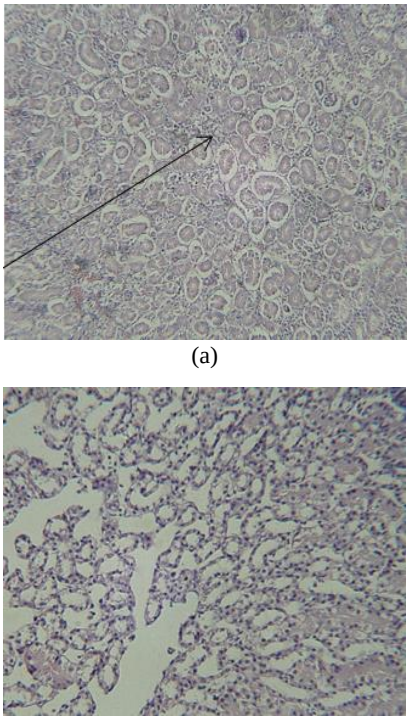
Removed kidney tissues were cut into small pieces resulting in homogenate and were freeze-thawed 3 times. RNA from the tissues was extracted and RT-PCR was performed. The result showed that the detection of the IB viral RNA from the renal tissues was 33.3%. After inoculation of the infected tissue into the chicken embryo followed by harvesting the allantoic fluid, the RT-PCR test results were positive (Fig. 4).

Table 1: Calculation of EID_{50} of XDC-2 strain of infectious bronchitis virus

	Chick embryo		Accumulated			
Virus dilution	Deaths	The number of healthy chicks	Dead	Live	Deaths/total	Mortality %
Total 10^{-2}	4	0	15	0	15/15	100
10^{-3}	4	0	11	0	11/11	100
10^{-4}	4	0	7	0	7/7	100
10^{-5}	3	1	3	1	3/4	75
10^{-6}	0	4	0	5	0	0

Table 2: Death status after induced infection of XDC-2 strain of IBV

Group	Virus dilution	Total	Incidence	Deaths	Mortality %
1	10^3 EID_{50}	6	6	1	16.7
2	10^3 EID_{50}	6	6	2	33.3
Control	PBS	5	0	0	0.0



(a)

(b)

Fig. 3: Pathological changes in renal tissues; (A) Dead chicken showed tubular dilatation, hyaline degeneration of epithelial cells, most of the glass cylinder lumen, and necrosis of epithelial cells; (B) Chicken from the control group showed normal renal tissues

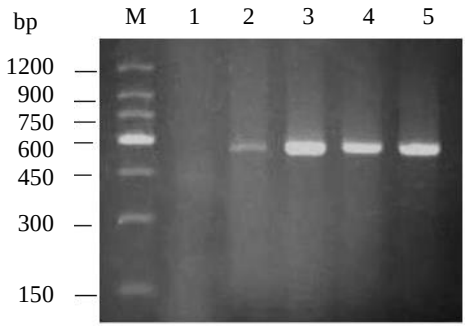


Fig. 4: RT-PCR of the allantoic fluid

Discussion

Infectious Bronchitis Virus (IBV) is prevalent in all countries with an intensive poultry industry. Respiratory disease is the most frequently observed syndrome caused by IBV (Cavanagh *et al*., 1997; Ji *et al.*, 2020).

IBV infection was evaluated by the embryo lesions/mortality method and confirmed by the RT-PCR method. Contrary to what might be expected, RT-PCR of allantoic fluid gave a lower proportion of infected birds than did observation of embryo lesions/mortality. This apparent contradiction may be explained by the variation between individual embrocated eggs in each test and the sampling method used. A result was considered positive when lesions/mortality were recorded in at least one of three injected eggs, whereas RT-PCR was performed on the pooled allantoic fluid harvested from two different eggs. At low virus levels, as may occur in the vaccinated/challenged chickens, there may be an uneven particle distribution in the injected eggs.

Studies conducted in Belgium and Italy showed that vaccines of the Mass serotype protected poorly against NIBV isolates (Boursnell *et al*., 1987; Cavanagh, 2007; Fabricant, 1998). Besides the heterogeneity of the vaccine and challenge viruses, insufficient renal protection can be attributed to the high level of attenuation of H120, resulting in a mild vaccine, which is sufficient to protect the respiratory tract but not sufficient to protect the kidney (Li *et al.*, 2022; Ma *et al*., 2019). A recent study conducted by Chen *et al.* (2021); Lin *et al*. (2016) showed that the use of a combination of two vaccines, Ma5 (Mass) and 4/91, was highly prophylactic against kidney damage caused by the homologous Belgian NIBV isolate B1648. This combination was not tested with other NIBV isolates.

The incidence of pathological changes in chicken lungs and kidneys was significant. The results indicated that this strain has a strong renal tissue tropism. Pathological examination revealed that the isolate caused both kidney and respiratory damage, with typical pathological features of airway epithelial cell damage, interstitial hyperemia, hemorrhage, large number of

lymph cell infiltration in pulmonary tissues, tissue necrosis, and infiltration of lymphocytes in kidney tissues. The control group had no abnormal tissues or organs.

RT-PCR has been successfully applied to identify IBV in many laboratories, PCR method is specific, sensitive, simple, and quick benefits (McFarlane and Verma, 2008). This study demonstrated that the XDC-2 strain of IBV has much tropism in the renal tissues. Virus isolation using embrocated chicken eggs and detection of the virus by PCR are quite useful for the diagnosis and identification of new IBV strains.

Conclusion

A nephritogenic strain of infectious bronchitis virus was successfully identified in some northern provinces of Vietnam and the histopathological changes showed tubular dilatation, hyaline degeneration of epithelial cells, most of the glass cylinder lumen, and necrosis of epithelial cells.

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Author's Contributions

Tran Duc Hoan: Conception, experiments, wrote and revised of the manuscript.

Doan Thi Thao, Nguyen Dinh Nguyen, Wang Bin and Nguyen Luan: Performed the experiments and collected the data.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues are involved.

Conflict of Interest

All authors confirm that we have no conflict of interest.

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