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Pathogenicity differences between QX-like and Mass-type infectious bronchitis viruses

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ABSTRACT

Infectious bronchitis is a highly contagious, acute viral respiratory disease of chickens, caused by infectious bronchitis virus (IBV). In recent years, the isolation rate of QX-like IBV has increased in the world. To clarify this phenomenon and better understand the pathogenicity of QX-like IBV, we examined differences in pathogenicity between two IBV strains, SD and M41, which belong to QX-like and Mass-type IBV, respectively. SD strain was more virulent in 3-week-old specific-pathogen-free chickens than M41 strain causing higher mortality with severe renal lesions. The tissue distribution of the two virus strains was tested by real-time RT-PCR. The results showed that the viral genome copy numbers in the tissues of chickens inoculated with SD strain were higher than those in chickens inoculated with M41 strain, with the exception of the trachea and lung. This study indicates that there are tremendous differences in pathogenicity and tissue tropism between the QX-like strain and Mass-type strain. These findings may benefit the prevention of infectious bronchitis in the poultry industry in China.

1. Introduction

Infectious bronchitis is a highly contagious, acute viral respiratory disease of chickens, caused by infectious bronchitis virus (IBV), which leads to great losses in the poultry industry including reduced numbers and quality of eggs from layers, and poor weight gain and feed efficiency in commercial broilers (Saif, 2003). Furthermore, some virulent strains of IBV can cause high mortality rates because of renal disease (Raj and Jones, 1997; Feng et al., 2012). Infected chickens are also susceptible to secondary infections with mycoplasma, bacteria or other pathogens because of tracheal cilia damage, leading to higher rates of mortality (Chhabra et al., 2015). Young chickens are the most susceptible to IBV. IBV not only affects the respiratory tract, but also causes damage to the digestive system and urogenital system, such as the proventriculus, kidney, ovary and oviduct, resulting in respiratory disease, interstitial nephritis and dysplasia of the oviduct (Raj and Jones, 1996; Benyeda et al., 2009; Zhong et al., 2016). Sneezing, tracheal rales and increased drinking are the main clinical signs. Lesions in infected chickens include degeneration of the renal epithelium, renal interstitial inflammation and necrosis of the ciliated respiratory epithelium.

IBV was first isolated in 1930 in the USA (Schalk and Hawn, 1931). Since then, several genotypes identified as the Massachusetts (Mass)-type of IBV have been reported (Jackwood, 2012). IBV became widespread from 1991 in China, and a new IBV variant has been prevalent in China since 1997, which was identified as the QX strain (Zhao et al., 2015). A previous study showed that the isolation rate of QX-like IBV has increased from 11.7% to 70% in the past 20 years in China, whilst the vaccine-like genotype IBV has decreased in prevalence from 50.4% to 4.4% (Zhao et al., 2016). The lack of cross protection among different IBV genotypes and the constant appearance of new IBV genotypes have been a serious challenge to the prevention and control of infectious bronchitis (Bande et al., 2015; Sjaak et al., 2011; Thor et al., 2011).

We previously isolated an IBV strain, designated "SD", from IBV-vaccinated chicken flocks. Phylogenetic analysis showed that this SD strain belonged to the QX-like genotype. Cross-neutralization assays revealed major serological differences between the SD and M41 strains (Yan et al., 2017). Here, we performed experiments to investigate the pathogenicity and tissue tropism of the SD and M41 strains, which belong to QX-like and Mass-type genotypes, respectively.

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2. Materials and methods

2.1. Virus

The QX-like IBV SD strain (GenBank accession no: KY421673) was isolated from chicken flocks vaccinated with Mass-type IBV vaccines in 2013 that showed obvious respiratory symptoms and renal disease. The M41 strain of IBV was kept in the laboratory. These two IBV strains were purified and propagated by inoculating 10-day-old specific-pathogen-free (SPF) chicken embryos *via* the allantoic cavity. Inoculated chicken embryos were incubated at 37 °C for 40 h and the allantoic fluid was collected for further study. The 50% embryo infectious doses (EID_{50}) were determined as previously described (Reed and Muench, 1938) by inoculating serial 10-fold allantoic fluid dilutions into SPF chicken embryos.

2.2. Animals and ethics statement

Three-week-old SPF chickens were purchased from the Beijing Merial Vital Laboratory Animal Technology Co., Ltd. (China), to compare the pathogenicity differences between the SD and M41 strains. The chickens were divided into three groups of 20 chickens each in isolators at China Agricultural University. Throughout the experiments, feed and water were available *ad libitum*. The treatment of all laboratory animals was approved by the Beijing Administration Committee of Laboratory Animals under the leadership of the Beijing Association for Science and Technology (approval ID SYXK [Jing] 2013-0013). The protocols for this experiment were performed according to the guidelines of the Animal Welfare and Ethical Censor Committee at China Agricultural University.

2.3. Pathogenicity experiments

Sixty 3-week-old SPF chickens were randomly divided into three groups of 20 chickens each and were housed in three isolators under positive pressure. Of the three groups, two were inoculated with 200 µl of SD or M41 strain containing 10^6 EID_{50} *via* the intraocular and intranasal routes. The third group was inoculated with normal saline as a negative control. All chickens were observed for 14 days, and food and water were provided *ad libitum* during the experiment.

2.4. Clinical observations and sampling

To determine the pathology of the two IBV strains, all chickens were observed daily for 14 days post-inoculation (dpi). Clinical signs caused by IBV consisted of depression, emaciation, cough, sneeze and tracheal rales. Two chickens from each group were euthanized at 1, 3, 5, 7 and 10 dpi. Gross lesions were noted and samples of the trachea, lung, proventriculus, spleen, duodenum, kidney and bursa of Fabricius were collected for virus load detection by real-time fluorescent quantitative reverse transcription polymerase chain reaction (RT-qPCR) and saved in 10% neutral formalin for histopathological examinations. Samples of the trachea were collected for detecting inhibition of ciliary activity caused by IBV.

2.5. Inhibition of ciliary activity

To evaluate tracheal ciliostasis, three rings of the upper, middle and lower part of the trachea, respectively, nine rings per chicken in total, were collected. Tracheal rings were placed in a 96-well plate containing Eagle's culture medium with 10% fetal bovine serum. They were then examined under an inverted light microscope at a magnification of 400× to observe the percent integrity of tracheal ciliary movement. Sections were scored as: 0, if all of the tracheal cilia showed movement; 1, if 75%–100% of the tracheal cilia showed movement; 2, if 50%–75% of the tracheal cilia showed movement; 3, if 25%–50% of the tracheal cilia showed movement; and 4, if less than 25% of the tracheal cilia showed movement or no movement at all was detected. The average score was calculated for each group.

2.6. Histopathology and immunohistochemistry (IHC)

The tissues collected as described above, were dipped into 10% neutral formalin for 48 h. Fixed samples were processed, embedded in paraffin wax, and cut into 5-µm sections. Sections were stained with eosin and hematoxylin, and examined by light microscopy for lesions. The same tissue sections were prepared for IHC to detect the intensity of viral antigen. The 5-µm sections were subjected to antigen retrieval and blocked by 10% normal goat serum in PBS for 30 min to eliminate nonspecific binding. Then sections were incubated with chicken anti-IBV hyperimmune serum at a 1:500 dilution in PBS for 12 h at 4 °C followed by incubation with horseradish peroxidase-conjugated rabbit anti-chicken IgG for 1 h. The reaction was visualized by 3,3-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) for 10 min. Finally, sections were counterstained with hematoxylin and examined by light microscopy.

2.7. IBV detection by real-time quantitative PCR

Total RNA of the following tissue samples was extracted using the RaPure Total RNA Kit (Magen, Beijing, China) according to the manufacturer's instructions: trachea, lung, proventriculus, spleen, kidney and bursa of Fabricius. Then, 500 ng of purified RNA was used as a template for reverse transcription with the PrimeScript™ RT Master Mix (TaKaRa Otsu, Shiga, Japan): 37 °C for 15 min, followed by 85 °C for 5 s. The cDNA obtained was analyzed by SYBR Green I real-time RT-qPCR to detect the viral load. Primers were designed based on the conserved regions of the *N* gene of SD and M41 strains using Primer Premier 5.0 (N-F 5′ AGTTTGAAGGTAGCGGTGTT 3′, N-R 5′ GTTAGC GGCTGGTCCTGT 3′). We inserted the fragment into the pEASY-Blunt vector to generate a standard plasmid as previously described (Zhao et al., 2015).

The RT-qPCR mixture was composed of 10 µl SYBR Premix Ex Taq (Takara), 0.4 µl of forward primer (10 mM), 0.4 µl of reverse primer (10 mM), 7.2 µl of nuclease-free water and 2 µl of cDNA (or nuclease-free water for the control). The thermal profile for the RT-qPCR was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 20 s, then melting at 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s using the Light Cycler 96 Real-Time PCR system.

The standard plasmid with the same region of the *N* gene inserted was subjected to RT-qPCR using serial dilutions (10^{-3}–10^{-9}) of the template as a quantification assay. We generated a standard curve by plotting the cycle threshold (CT) values against the copy numbers of the standard plasmid with each dilution.

2.8. Statistical analysis

Statistical analysis of the RT-qPCR, survival and ciliostasis scores were performed using Prism 6.0 program (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was considered as follows: significant at $P \leq 0.05$ (*); highly significant at $P \leq 0.01$ (**); and very highly significant at $P \leq 0.001$ (***).

3. Results

3.1. Clinical signs

Chicks inoculated with the SD strain began to show clinical signs on the third day. Diseased chicks showed signs of sneezing, listlessness and huddling. Four chickens (20%) in the SD inoculated group died during the experiment observation period. In the M41 inoculated group, chicks only showed signs of sneezing and slight listlessness, no chicks died.