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Research paper

Phylogenetic and antigenic analysis of avian infectious bronchitis virus in southwestern China, 2012–2016

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ABSTRACT

The aim of this study was to decipher the molecular epidemiological and antigenic characteristics of infectious bronchitis virus strains (IBVs) isolated in recent years in southwestern China. A total of 24 field strains were isolated from diseased chickens between 2012 and 2016. Phylogenetic analysis based on S1 nucleotide sequences showed that 16 of the 24 isolates were clustered into four distinct genotypes: QX (37.5%), TW (16.7%, TWI and TWII), Mass (8.3%), and J2 (4.2%). The QX genotype was still the prevalent genotype in southwestern China. Recombination analysis of the S1 subunit gene showed that eight of the 24 field strains were recombinant variants that originated from field strains and vaccine strains. A new potential recombination hotspot [ATTTT(T/A)] was identified, implying that recombination events may become more and more common. The antigenicity of ten IBVs, including seven field strains and commonly used vaccine strains, were assayed with a viral cross-neutralization assay in chicken embryonated kidney cells (CEK). The results showed that the ten IBVs could be divided into four serotypes (Massachusetts, 793B, Sczy3, and SCYB). Sczy3 and 793B were the predominant serotypes. Six of the seven field isolates (all except for cK/CH/SCYB/140913) cross-reacted well with anti-sera against other field strains. In conclusion, the genetic and antigenic features of IBVs from southwestern China in recent years have changed when compared to the previous reports. The results could provide a reference for vaccine development and the prevention of infectious bronchitis in southwestern China.

1. Introduction

Infectious bronchitis (IB) is a highly contagious disease in chickens that causes significant economic losses to the worldwide poultry industry (Colvero et al., 2015). The etiologic agent of IB is the infectious bronchitis virus (IBV), a member of the Coronaviridae family, in the subfamily coronaviridae and genus gamma-coronavirus. The IBV genome is 27.6 kb and encodes at least four structural proteins, including the spike glycoprotein (S), membrane protein (M), small membrane protein (E), and nucleocapsid protein (N) (Ujike and Taguchi, 2015). The major immunogen of IBV is the S1 subunit protein, which contains epitopes that can induce the production of specific neutralizing antibodies and the hemagglutination inhibition antibody. IBVs from different serotypes usually exhibit poor cross-protection (Li et al., 2012). Due to the incomplete proofreading mechanism of the RNA polymerase and the gene recombination during genome replication, IBV genomes are constantly evolving, and new IBV variant strains are always arising (Baker and Lai, 1990; Lai, 1992).

Since the early 1980s, IBV has been diagnosed in China by viral isolation. Although the wide use of vaccine strains, such as H120, M41, 28/86, 4/91, and ma5, has successfully prevented IB epidemics on most farms, immune failure is still reported frequently as the result of infections with strains that differ serologically from the vaccine strains. Therefore, continuing analysis of the genetic evolution and antigenic relatedness among field isolates and vaccine strains may provide critical insight for vaccine strain selection and vaccine development. Our previous study revealed that isolates obtained between 2008 and 2009 from the Sichuan province belonged mainly to a group of QX-like strains (79% QX-type; 5% TWI-type) (Zou et al., 2010). In a later report from other researchers, QX-type and TWI-type IBVs accounted for 37% and 37%, respectively, in Sichuan area during 2011–2012 (Zhang et al., 2016). While in Southern China, picture was quite different, cK/CH/LSC/99I-type was the predominant genotype and no QX-type strains were isolated during 2013 (Mo et al., 2013). So the genetic character of IBVs from China varied according to time and regions. For the antigenic features of IBVs isolated in recent years from China, less attention was given. A report showed that 28 IBVs from Guangxi of China in 2009–

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2011 could be divided into 6 serotypes (I-VI), but most of the isolates (24/28) was QX-genotype, IBVs from the QX-genotype may belong to different serotypes, and the serotype of IBVs varied according to time and regions (Qin et al., 2014). Another report showed the serotype of TWI-type strains were different from Mass-type in Taiwan in 2000 (Wang and Huang, 2000). As the genetic and antigenic character of IBVs varied according to time and regions, and there is no official report on that of the IBVs from southwestern China in recent years, the molecular and antigenic characteristics of IBVs from southwestern China were not clear. The aim of this study was to decipher the genetic and antigenic characteristics of IBV strains circulating in commercial flocks in southwestern China in recent years.

2. Materials and methods

2.1. Eggs and virus

Specific pathogen-free (SPF) chicken embryos were obtained from Beijing Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China). M41 and H120 strains were obtained from the China Institute of Veterinary Drug Control (Beijing, China). The 4/91 vaccine was supplied by Internet International B.V. (Boxmeer, NL).

2.2. Viral isolation

Throughout 2012–2016, kidney, lung, and trachea samples were collected from broiler or layer chickens suspected of IB infection in southwestern China (Table1). Samples were homogenized in phosphate-buffered saline (PBS) containing 200 μg/mL penicillin and 100 μg/mL streptomycin in a ratio of 1:5–10. After filter sterilizing with a 0.22 μm filter membrane, 0.2 mL sample was inoculated into the allantoic cavity of 9- to 11-day-old SPF embryos. The embryos were incubated at 37 °C and examined twice daily for their viability. The allantoic fluids were harvested after 36 h incubation, and three blind passages were conducted. The presence of IBV was verified by reverse transcription-polymerase chain reaction (RT-PCR) of the N gene (Zou et al., 2010). The existence of other five pathogens, H9 subtype Avian influenza virus (H9 AIV), Newcastle disease virus (NDV), Marek's disease virus (MDV), bacteria and Coccidiosis in those samples were verified by following the methods of other reports (Abu-Akkada and Awad, 2012; Chen et al., 2012; Li et al., 2010; Rui et al., 2010; Tian et al., 2011).

2.3. Phylogenetic and recombination analysis of the S1 genes

Total RNA was extracted from IBV-infected allantoic fluid with RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions and dissolved in 40 μL sterile diethylpyrocarbonate (DEPC)-treated water before being stored at −70 °C for further use. For the reverse transcription (RT) reaction, 5 μL of template RNA, 2 μL of 5× RT Mix, and 3 μL of RNase-free water were added and mixed. The reaction mixture was incubated at 37 °C for 15 min and then at 85 °C for 1 min. PCR amplification and cloning of the S1 gene was performed as the previous report (Zou et al., 2010). The recombinant plasmids containing the target gene were sequenced by Shanghai Sanggong Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Nucleotide sequences of the S1 gene obtained from the IBV isolates were aligned using the Editseq program in the Lasergene package (DNASTAR Inc., Madison, WI, USA) and compared to the sequences of 53 other reference IBVs using the MegAlign program in the same package. For the 53 reference IBVs, 43 strains were isolated from China, 2 strains were isolated from the USA, 2 strains were from Japan, and the other 6 strains were vaccine strains. A phylogenetic tree of the S1 gene was created using the neighbor-joining method in MEGA version 7.0.14. Bootstrap values were determined from 1000 replicates of the original data.

The S1 subunit sequences of 24 IBV field strains and 53 reference strains were aligned by MegAlign, and putative recombinant strains were selected by sequence homology analysis. In order to identify the assumed parent sequences, the S1 subunit sequences of suspected recombinant isolates were blasted against the GenBank database of the National Center for Biotechnology Information (NCBI). Recombination analysis of the selected sequences was conducted with the aid of Recombination detection program (RDP 4.72) and SimPlot version 3.5.1 software. Potential recombination events were identified using the RDP, Maxchi, and GENECONV methods in RDP 4.72 to identify putative parental sequences with significance set at P values <0.05 and the sliding window size set as 30 bp. Putative potential recombination events were further identified using the SimPlot version 3.5.1. Nucleotide identities were calculated using the Kimura 2-parameter method with a transition-transversion ratio of 2 in each window of 200 bp, and the window was successively extended in 20-bp increments.

2.4. Viral cross-neutralization test

For the preparation of antisera against the ten IBVs, 8-week old rabbits ($n = 4$) were immunized with purified 10^4 EID_{50} IBVs subcutaneously mixed with an equal volume of complete Freund's adjuvant (Sigma, Missouri, USA) for the first injection, and with the same antigen emulsified in freund's incomplete adjuvant for the following two booster injections (two-week interval). Rabbits were held in separate biosafety level 2 (BSL2) isolators in the Laboratory Animal center of Sichuan Agricultural University (Ya'an, Sichuan, China) with a libitum access to feed and water and maintained under uniform standard management conditions. Approval for these animal studies was obtained from the Sichuan provincial Laboratory Animal Management Committee [Permit Number: XYXK(Sichuan) 2014-187] and the Ethics and Animal Welfare Committee of Sichuan Agricultural University. Antisera from vaccinated animals were collected at 12 days after the final immunization and stored at −20 °C.

To determine the antigenic relatedness between the field IBV isolates and the vaccine viral strains, double-direction viral cross-neutralization (VN) tests were performed in chicken embryo kidney (CEK) cells using constant viral titers and diluted serum. The tested strains came from six different genotypes and included seven IBV field isolates (Sczy3, cK/CH/SCDY/141030, cK/CH/SCLS/140104, cK/CH/CQKX/150203, cK/CH/SCYB/140913, cK/CH/SCMY/10I, cK/CH/SCYB/141102) and the three most commonly used vaccine viral strains (H120, M41, and 4/91).

Before VN testing, IBV strains were adapted to CEK cells by serial passaging. Briefly, allantoic fluid containing the IBV strain was propagated in monolayer primary CEK cells prepared from 18- to 20-day-old chicken embryos. Infected CEK cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 2% fetal bovine serum (Zhejiang Tian-hang Biological Technology Stock Co., Ltd., Zhejiang, China) and incubated at 37 °C with 5% CO_2. The supernatant was harvested 40 h post-inoculation and passaged blindly in CEK cells until a characteristic cytopathic effect (CPE), such as syncytia, was observed. Determination of the $TCID_{50}$ of the CEK-adapted IBVs in CEK cells was conducted per the method of Reed and Muench (1938).

For the VN test, equal volumes of 100 $TCID_{50}$ of the CEK-adapted IBVs and serial two-fold dilutions of antisera were mixed and kept at 37 °C for 1 h. Next, 0.4 mL of the virus–antisera mixture was then transferred to CEK cell cultures in 24-well plates (6 wells for each dilution). The plates were incubated for 72 h, and the 50% end-point neutralizing titers were calculated by the method of Reed and Muench (1938). Negative rabbit serum was also incubated with 100 $TCID_{50}$ of IBV to calculate its non-specific neutralizing titer to IBVs, and this neutralizing titer was used as a background value for further analysis.

The VN end-point titers were used to calculate the antigenic relatedness values (ARV, r) by the method of Archetti & Horsfall