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Research Paper

Genetic diversity of avian infectious bronchitis virus in China in recent years

Liwen Xu, Zongxi Han, Lei Jiang, Junfeng Sun, Yan Zhao, Shengwang Liu*

Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin 150001, People's Republic of China

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ABSTRACT

In this study, 213 infectious bronchitis viruses (IBVs) were isolated from samples collected from 801 flocks suspected to be infected with IBV from January 2016 to December 2017 in China. By using complete nucleotide sequences of S1 gene we determined the phylogeny of these IBV isolates, which in turn allowed us to define six lineages/genotypes, a number of recombinants and a novel variant. The GI-19 lineage was the most frequently isolated type in China in recent years. Although scattered mutations in the *S1* gene among the GI-19 lineage viruses were observed, we also noted different sublineages in the GI-19 lineage with unique mutations, suggesting a high degree of *S1* gene variation since they were first isolated in the mid-1990s. We also isolated a number of vaccine-like viruses from vaccinated diseased chickens, although more work is needed to differentiate the reisolation of vaccine strains from field strains of the same serotype. One of the important findings in this study is that the prevalence of the TW I type viruses in GI-7 lineage has been increasing in recent years in China. Another important finding is that recombination events occurred between the predominant GI-19 lineage and the commonly used 4/91 vaccine, which gave rise to distinct IBV isolates. In addition, a novel IBV isolate, together with a reference strain in GenBank database, were found to form a novel lineage/genotype that was remarkably distinct from established lineages. The characteristics of the antigenicity, tissue tropism, pathogenicity and complete genome were required for further investigation for the recombinants and the viruses in different sublineages and novel lineages. Meanwhile, permanent monitoring of circulating strains was needed to monitor the emerging viruses and rationally modify vaccination strategies in the field situation.

1. Introduction

Infectious bronchitis virus (IBV) is a member of the species Avian Coronavirus, genus Gammacoronavirus, and affects the respiratory tract, kidneys, gut and oviduct, and IBV infection is among the most important poultry diseases worldwide (Cavanagh, 2007). In most cases, the birds show respiratory problems such as nasal and/or ocular discharge and other respiratory distress, poor performance, decreased egg production, poor egg shell quality, reduced hatchability, nephritis and sometimes false layers, however, the clinical signs caused by IBV infection can be diverse and nonspecific. The infection is often complicated by secondary opportunistic agents such as *Escherichia coli* (Dwars et al., 2009; Vandekerchove et al., 2004), *Mycoplasma gallisepticum* (Georgiades et al., 2001) and *Ornithobacterium rhinotracheale* (Vandekerchove et al., 2004), and combinations of IBV with other pathogens can seriously increase the level of damage (Haghighat-Jahromi et al., 2008).

IBV has a linear, single-stranded RNA genome of positive polarity of ~27 kb in length and produces enveloped virions. IBV particles consist of four structural proteins: spike (S) glycoprotein, membrane (M), small envelope (E) and nucleocapsid (N) proteins. The S protein is formed by post-translational cleavage of S into two separate polypeptide components, S1 and S2 (Cavanagh, 1983, 2007). S1 mediates virion attachment to host cells (Casais et al., 2003) and is a target of neutralizing antibodies in chickens (Cavanagh et al., 1986). Mutation and recombination in the genome, especially in the *S1* gene, may give rise to variants or new genotypes that may escape from the protection offered by the immunity induced by the classical vaccination programs (Cavanagh, 2007). Hence, genotyping of IBV is associated primarily with changes in the amino acid sequence of the S1 subunit (Cavanagh et al., 1988; Kant et al., 1992), and evolutionary characterization of IBV is mainly based on analysis of the S1 gene (Lee et al., 2003; Wang et al., 1994).

Different IBV genotypes are distributed globally. Some of them are endemic only in particular countries or region, while others circulate worldwide (De Wit et al., 2011). Six genotypes that together comprise 32 distinct viral lineages and a number of interlineage recombinants have been defined worldwide by phylogenetic analysis with the

* Corresponding author.
E-mail address: liushengwang@caas.cn (S. Liu).

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complete nucleotide sequences of the *S1* gene (Valastro et al., 2016). Recently, two additional types of IBV that are genetically distinct from those of the 32 lineages and interlineage recombinants were found in China by phylogenetic analysis of complete nucleotide sequences of *S1* genes of a number of IBV isolates (Chen et al., 2017; Jiang et al., 2017). They were defined as lineages 28 and 29 in genotype I (GI-28 and GI-29, respectively). This large diversity of lineages/genotypes is a major reason why commercial vaccines often fail or only provide partial protection against IBVs, and therefore, new IB outbreaks continue to occur in vaccinated chicken flocks.

In China, several epidemiological IB surveys have already been conducted and have shown that a variety of IBV genotypes and variants (Liu et al., 2013; Mo et al., 2013; Zhao et al., 2013; Liu et al., 2014; Zhou et al., 2014; Chen et al., 2015; Xu et al., 2016; Zhang et al., 2015; Leghari et al., 2016; Chen et al., 2017; Zhao et al., 2017; Zhou et al., 2017) are circulating and cocirculating. This shows that the Chinese IBV situation is complex and variable, and causes serious economic losses in the Chinese poultry industry. In addition, IB outbreaks also occur in well-vaccinated flocks using different serotype vaccines and vaccination programs. This raises the question of whether new IBV variants, against which vaccines induce an insufficient level of cross-protection, are involved. In our conventional surveillance of IB between January 2016 and December 2017 in China, broilers and layer chickens with suspected IB with associated respiratory problems, nephritis and reduced egg production frequently occurred. In the present study, cloacal swabs and tissue samples were collected from 211 and 590 commercial chicken flocks, respectively. Two hundreds and thirteen IBVs were isolated and the complete nucleotide sequence of *S1* genes were sequenced and analyzed. The aims of the study were to gather recent data, update knowledge of the circulating IBV field strains, and obtain a wider picture of IBV genetic diversity to understand better the epidemiology of IBVs circulating in chicken flocks in China in recent years.

2. Materials and methods

2.1. Samples, virus isolation and purification

A total of 801 flocks from 17 provinces (cities) were involved in this study (Supplemental Table 1 and Fig. 1), during the 2 years of IB surveillance in China. Sampling was conducted from early January 2016 to the end of December 2017. A wide variety of IB vaccination programs had been used in the chickens from which the samples were collected. In all cases, Massachusetts (Mass) type H120 or Ma5 vaccines had been used at 1 day old by coarse spray or oculonasal route. A second vaccination with 4/91 or other vaccine, such as LDT3-A, was also common between 15 and 20 days old. From a given flock on each farm, > 20 cloacal swabs were collected. Tissue samples from the trachea, lungs and kidneys were obtained from more than three birds from each flock. The cloacal swabs or tissue samples from the same flock were pooled and used for viral isolation. Hence, a pool of cloacal swabs or tissues represented one sample. The cloacal swabs were placed in 1 ml buffered peptone water and kept at 4 °C until use.

The pooled tissue samples were homogenized at a ratio of 1:10 *w/v* in 1 ml diethyl-pyrocarbonate-treated phosphate-buffered saline in a TissueLyser (Qiagen, Hilden, Germany). Tissue homogenates or cloacal swabs were clarified at 1000 × g for 10 min at 4 °C. Viral isolation was performed by inoculation of 9-day-old embryonated, specific pathogen-free (SPF) hens' eggs (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences) via the allantoic cavity route with the supernatant from the homogenates or cloacal swabs. For each sample, three eggs were used for viral isolation. Eggs were incubated at 37 °C and candled daily (Liu and Kong, 2004). Embryos that died within 24 h after inoculation were discarded. On day 3 after inoculation, 100 μl allantoic fluid was collected from each egg, using a 26 gauge needle, through a small hole created in the shell near the air sac (Hewson et al., 2012). The allantoic fluids from the three eggs were pooled and 50 μl pooled allantoic fluids were used to detect IBV using RT-PCR assays with two set of primers targeting two conserved regions in the IBV genome. The remaining allantoic fluids were blind passaged 3–8 times in SPF eggs until the typical embryonic lesions of IBV infection were observed. The eggs were incubated for further 4 days after the allantoic fluids were collected and observed for typical embryonic lesions of IBV infection, such as dwarfing, stunting, and curling, with feather dystrophy.

For the virus-infected allantoic fluids containing inconsistent S1 sequences, each type of virus was isolated by purifying the allantoic fluids using limiting dilution in embryonated SPF eggs. Serial 10-fold dilutions of the allantoic fluids were inoculated into the allantoic cavity of SPF chicken embryos, which were observed for 2 days. Purification of the virus was identified by S1 amplification, cloning and sequencing of at least 10 clones of each virus. The allantoic fluids of the eggs inoculated with the highest dilution that had consistent S1 sequences were used for another two rounds of purification.

2.2. Eggs

White Leghorn SPF fertile chicken eggs were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, China. All experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang province, China.

2.3. RNA extraction and RT-PCR

The virus passages used for RNA extraction were listed in the Supplemental Table 1. RNA was extracted from the allantoic fluids using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was eluted in 30 μl RNase-free water and was used as the template for RT-PCR amplification.

Two sets of oligonucleotide pairs, one targeting the *N* genes [N(+) and (N−)] and another targeting a region of the viral genome downstream of the gene *5* and upstream of *N* (IBV-170 and IBV-171) (Liu et al., 2013), were used for IBV detection in allantoic fluids. The primers, S1Oligo5' and S1Oligo3′ (Kwon et al., 1993), were used for amplifying the complete *S1* genes of the IBV isolates. For the IBV-infected allantoic fluids that were negative for S1Oligo5' and S1Oligo3' by RT-PCR, the sequences upstream of *S* gene and downstream of the cleavage site of S proteins were determined (Zhao et al., 2017). New primers were designed based on the newly determined sequences and used for RT-PCR amplification and subsequent *S1* gene sequencing.

The RT-PCR was performed using the One-step RT-PCR kit (Takara Bio, Shiga, Japan) under the following conditions: reverse transcription reaction for 30 min at 50 °C; denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. Upon amplification, 20 μl PCR products were run in 1.5% agarose gel electrophoresis and visualized by GelGreen Nucleic Acid Gel (TaKaRa Bio) staining; bands of the expected size were excised from the gel for sequencing using the Gene Clean Kit (ExoSAP-IT, Affymetrix, Santa Clara, CA, USA). Purified PCR products were cloned into a pMD 18-T vector (TaKaRa Bio). Five to 10 clones were subjected to Sanger sequencing for each of the genes.

2.4. Sequence analysis

The obtained chromatograms were manually checked and edited for unclear base calls. Assembly of consensus nucleotide sequences was conducted using the BioEdit version 7.2.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The specific identification was primarily conducted by comparing the obtained sequences to on-line available reference sequences by BLAST searching (https://blast.ncbi.nlm.nih.gov/Blast). Sequences of *S1* genes from reference IBV strains with different genotypes/lineages were selected and retrieved from NCBI GenBank

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