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Research paper

Genome characterization, antigenicity and pathogenicity of a novel infectious bronchitis virus type isolated from south China

Lei Jiang, Wenjun Zhao, Zongxi Han, Yuqiu Chen, Yan Zhao, Junfeng Sun, Huixin Li, Yuhao Shao, Liangliang Liu, Shengwang Liu*

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

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ABSTRACT

In 2014, three infectious bronchitis virus (IBV) strains, designated as γCoV/ck/China/I0111/14, γCoV/ck/China/I0114/14 and γCoV/ck/China/I0118/14, were isolated and identified from chickens suspected to be infected with IBV in Guangxi province, China. Based upon data arising from S1 sequence and phylogenetic analyses, the three IBV isolates were genetically different from other known IBV types, which represented a novel genotype (GI-29). Virus cross-neutralization tests, using γCoV/ck/China/I0111/14 as a representative, showed that genotype GI-29 was antigenically different from all other known IBV types, thus representing a novel serotype. Complete genomic analysis showed that GI-29 type viruses were closely related to and might originate from a GX-YL5-like virus by accumulation of substitutions in multiple genes. These GI-29 viral genomes are still evolving and diverging, particularly in the 3′ region, although we cannot rule out the possibility of recombination events occurring. For isolate γCoV/ck/China/I0114/14, we found that recombination events had occurred between nsps 2 and 3 in gene 1 which led to the introduction of a 4/91 gene fragment into the γCoV/ck/China/I0114/14 viral genome. In addition, we found that the GI-29 type γCoV/ck/China/I0111/14 isolate was a nephropathogenic strain and high pathogenic to 1-day-old specific pathogen-free (SPF) chickens although cystic oviducts were not observed in the surviving layer chickens challenged with γCoV/ck/China/I0111/14 isolate.

1. Introduction

Infectious bronchitis virus (IBV) is a gammacoronavirus (family *Coronaviridae*, order *Nidovirales*) which causes highly contagious respiratory, reproductive, and urogenital symptoms in chickens (Cavanagh, 2007); consequently, this virus is of economic significance. This virus exists in a wide range of genetically and antigenically distinct types, making the prevention and control of this pathogen very challenging. While it is believed that the natural host of IBV is the chicken, the presence of IBV-like and other avian coronaviruses has been reported in both domestic and wide birds (Cavanagh, 2005, 2007), making the epidemiology and mechanisms of infection very complex.

The genomic architecture of coronaviruses, such as IBV, is characterized by two large overlapping open reading frames (ORFs), ORF1a and ORF1b, which encode components of the viral replicase and occupy approximately two-thirds of the genome. ORFs encoding the structural proteins, spike (S) glycoprotein, membrane (M) glycoprotein, nucleocapsid (N) phosphoprotein and envelope (E) protein, are located downstream of ORF1a/b and are expressed from 3′ co-terminal subgenomic mRNAs (Brian and Baric, 2005; Sawicki et al., 2007). The S protein is post-translationally cleaved into S1 and S2 fragments. The S1 subunit of S protein carries the receptor binding site and thus plays an important role in tissue tropism (Belouzard et al., 2012). The S1 protein is also the major inducer of neutralizing and serotype-specific antibodies and thus plays an important role in protective immunity (Wickramasinghe et al., 2011). In addition, S1 is the most variable gene among IBV isolates and such variability may lead to important biological differences between strains; novel serotypic variants could emerge as a result of amino acid changes in spike protein. Generally, the serotypes of IBV differ from each other by 20%–25% at the amino acid level in S1, but may differ by up to 50% (Cavanagh, 2007). In some cases, as little as 2% variation, or 10–15 amino acid changes in the amino acid sequence, can lead to a new IBV serotype because the epitopes eliciting neutralizing antibodies are widely distributed within the spike protein sequence (Cavanagh, 2007; de Wit et al., 2011a; Hodgson et al., 2004). Accordingly, analysis of the S1 gene has been

* Corresponding author.
E-mail address: swliu@hvri.ac.cn (S. Liu).

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conventionally used to determine viral genetic types (Valastro et al., 2016).

Currently, at least 50 different antigenic and genetic types of IBV that are poorly cross-protective have been discovered and studied by virus-neutralization tests and molecular characterization of the S1 protein gene (de Wit et al., 2011a, 2011b; Jackwood et al., 2012). Furthermore, new genotypes of IBV and associated variants appear frequently in different parts of the world; these have arisen because of mutations, especially in the S1 subunit, including point mutations, insertions, deletions, and also recombination between different IBV strains (Cavanagh et al., 1992; Jackwood et al., 2012). New genotypes often show antigenic variation and, hence, define new serotypes, and vaccinated poultry may still therefore be infected by serologically distinct strains of IBV. In addition, antigenic variants resulting in a reduced level of cross-protection between different strains represents a major obstacle for the development of an efficacious vaccine.

IBV was recorded in China for the first time in the 1980s (Han et al., 2011). Surveillance studies have reported widespread IBV infections in China since the late 1990s with associated multiple types; LX4 (QX-like) is the predominant type circulating in chicken flocks and first emerged in 1995 (Zhao et al., 2017) and at least five other serotypes (Chen et al., 2017; Gao et al., 2016; Han et al., 2011; Liu et al., 2007) and variants (Han et al., 2016; Liu et al., 2013, 2014; Ma et al., 2012) have been circulating in China over recent years.

In this study, we isolated three IBVs in local chickens from south China (Guangxi province) and investigated genotype, complete genomic characteristics, antigenicity and pathogenicity of each variant in order to understand the origin and evolution of this type of virus.

2. Materials and methods

2.1. Virus isolation

Three IBV viruses, γCoV/ck/China/I0111/14, γCoV/ck/China/I0114/14 and γCoV/ck/China/I0118/14, were isolated in Guangxi province from local yellow feature chickens which showed respiratory signs at 25-, 43- and 53-days-old, respectively. The flock where the γCoV/ck/China/I0118/14 was isolated from was located in Liuzhou, and the other two flocks where the γCoV/ck/China/I0111/14 and γCoV/ck/China/I0114/14 viruses were isolated were located in Guilin. Chickens from the three flocks were spray-vaccinated with IB vaccine H120 when they were 1-day-old followed by a H120 booster vaccination on day 7 of age by eye-drop inoculation. The viruses were isolated from the tracheas of diseased chickens using 9-day-old embryonated specific pathogen-free (SPF) chicken eggs as described previously (Liu and Kong, 2004). Viral titer was determined by titration in 9-day-old embryonated chicken eggs and calculated as previously described (Reed and Muench, 1938) to provide $10^{5.6}$ of the 50% egg infectious dose (EID_{50}) in 0.1 ml.

In addition, six IBV strains, including ck/CH/LDL/091022 (Liu et al., 2013), ck/CH/LGX/111119 (Chen et al., 2017), ck/CH/LDL/97I (Liu et al., 2007), ck/CH/LDL/140520 (Xu et al., 2016), 4/91 and H120 were used in this study. Strains ck/CH/LDL/091022, ck/CH/LGX/111119, ck/CH/LDL/97I and ck/CH/LDL/140520 represent the four major IBV serotypes circulating in chicken flocks in China. Strains 4/91 and H120 are IB vaccines commonly used in China. All of these viruses were propagated in 9-day-old embryonated SPF eggs and viral titer was determined as mentioned above.

2.2. Chickens and eggs

SPF White Leghorn chickens and fertile eggs from SPF chickens (Harbin Veterinary Research Institute, Harbin, China) were used in this study. Feed and water were free of any antibiotics. All of the experimental procedures were undertaken with the approval of the Harbin Veterinary Research Institute ethical review committee and according to Chinese legislation on the use of animals for experiments, as permitted under project license LHLJ20160321.

2.3. S1 gene sequencing and genotyping of the IBV isolates

A viral RNA extraction kit (TaKaRa Bio Inc., Shiga, Japan) was used to extract total viral RNA from infectious fluids in accordance with the manufacturer's instructions, and S1 genes from the three IBV isolates were amplified using S1Oligo5′ and S1Oligo3′ primers (Adzhar et al., 1997). Amplified PCR products were sequenced directly using sense and antisense primers and sequences were first analyzed with BLASTn (BLASTn: http://blast.ncbi.nlm.nih.gov/Blast.cgi). S1 genes from 96 reference IBV strains were selected and used for phylogenetic analysis with those of our three strains. Phylogenetic analyses were performed using the maximum likelihood method with the general time-reversible nucleotide substitution model and bootstrap tests of 1000 replicates in the MEGA 6 program (Tamura et al., 2013). In addition, pairwise comparison was conducted using S1 genes from 96 references strains and our three isolates according to the results of both BLASTn and phylogenetic analysis.

2.4. Complete genome sequencing and sequence analysis

Our strategy for sequencing the complete genomes of our three IBV isolates was the same as that described previously (Liu et al., 2013). Briefly, twelve overlapped primers were used to amplify the genomes of our three IBV isolates. The extreme 5′ and 3′ termini of the three isolates were determined by rapid amplification of cDNA ends (RACE), using a 3′/5′ RACE kit (Takara Bio Inc.,) according to the manufacturer's instructions. Each of the regions was sequenced at least three times. Nucleotide sequences were edited and assembled, and consensus full-genome sequences were determined and analyzed using the Clustal W method available in Bioedit software package v7.0.3.0. (http://www.mbio.ncsu.edu/bioedit/bioedit) by comparison with a reference, the complete genome of the Beaudette strain published in GenBank. The identities of complete genomic sequences between our three isolates were compared and calculated and then BLASTn was conducted using the complete genomic sequence of isolate γCoV/ck/China/I0111/14. The available complete genomic sequences from 21 reference IBV strains representing different serotypes/genotypes were selected and used for phylogenetic analysis with those of our three strains. Phylogenetic analyses were performed using the maximum likelihood method with the general time-reversible nucleotide substitution model and bootstrap tests of 1000 replicates in the MEGA 6 program (Tamura et al., 2013). Two sequences (GX-YL5 and GX-YL9), which showed the highest identities (97% and 96%, respectively) with the complete genome of our isolate γCoV/ck/China/I0111/14, two other sequences from the vaccine strains (4/91 and H120) which are commonly used in China, and one additional sequence from the predominant IBV serotype (ck/CH/LDL/091022) circulating in China, were selected and included in the alignment using Multiple Alignment with Fast Fourier Transformation (MAFFT) v6 (http://mafft.cbrc.jp/alignment/software/).

To further study the relationship between our three IBV isolates, we created a similarity plot (SimPlot) using a 500 bp window with a 50 bp step with the isolate γCoV/ck/China/I0111/14 as the query strain. Twelve fragments were obtained based upon the results of multiple sequence alignment constructed with MAFFT v6 and SimPlot. To further investigate the genetic relationship between our isolates and the reference strains, and the possibility of recombination events occurring during the origin of our isolates, we constructed maximum likelihood phylogenetic trees based upon the sequences of each fragment with the general time-reversible nucleotide substitution model and bootstrap tests of 1000 replicates in the MEGA 6 program (Tamura et al., 2013).

To further characterize recombinants, the data set was scanned using a Recombination Detection Program (RDP) v2 with the implemented algorithms GENECONV, BootScan, MaxChi, Chimera and

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