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Characterization of the complete genome, antigenicity, pathogenicity, tissue tropism, and shedding of a recombinant avian infectious bronchitis virus with a ck/CH/LJL/140901-like backbone and an S2 fragment from a 4/91-like virus

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ABSTRACT

In this study, we isolated an infectious bronchitis virus, designated I1101/16, from broiler breeders in China. Analysis of the S1 gene showed that isolate I1101/16 was genetically close to strain ck/CH/LJL/140901, which belongs to the TW I genotype (also known as lineage GI-7 based on the recent IBV classification), however the S2 gene showed genetic diversity comparing to that of S1 gene. Comparison of the genomic sequences showed that the genome of isolate I1101/16 was similar to that of strain ck/CH/LJL/140901 from the 5′ end of the genome to the 5′ end of the S2 gene and from the 5′ end of the 3a gene to the end of the genome, whereas the remaining parts of the genome sequences were more closely related to those of strain 4/91 than those of ck/CH/LJL/140901, thereby suggesting that recombination might have occurred during the origin of the virus. SimPlot and Bootscan analysis of the complete genomic sequence confirmed this hypothesis, where it showed that isolate I1101/16 arose through recombination events between ck/CH/LJL/140901- and 4/91-like viruses. Isolate I1101/16 and strain ck/CH/LJL/140901 shared identical amino acids in almost all five of their B cell epitopes, but the two viruses had a serotype relatedness value of 65, which is well below 80, i.e., the lower cutoff value for viruses of the same serotype. In addition, pathogenicity tests demonstrated that isolate I1101/16 was more pathogenic to 1-day-old specific-pathogen-free chickens than strain ck/CH/LJL/140901, according to analysis of the clinical signs, whereas strain ck/CH/LJL/140901 exhibited prolonged replication and shedding after challenge compared with isolate I1101/16. This study did not provide evidence that recombination can directly alter the antigenicity, virulence, replication, shedding, and tissue tropism of a virus, but it did show that recombination events are likely to be major determinants of viral evolution.

1. Introduction

Avian infectious bronchitis virus (IBV), the prototype *Gammacoronavirus* species in the family Coronaviridae, is a positive-sense, single-stranded RNA virus with a genome of approximately 27.6 kb (5′-untranslated region (UTR)-1a/1ab-S-3a-3b-E-M-5a-5b-N-3′-UTR). It causes an acute and highly contagious disease in chickens, which is responsible for high economic losses in the poultry industry. The current control strategies are based mainly on mass vaccination strategies. Nevertheless, vaccine-induced immunity generally give poor protection because the current vaccine offers only limited cross protection among strains (Cook et al., 2012; de Wit et al., 2011) mainly due to the antigenic diversity caused by the variability of the S1 protein. Recently, numerous IBV strains have been identified and new genotypes/serotypes have emerged from existing viruses via point mutations, insertions, and deletions in the viral genome, especially in the S1 subunit of the spike protein gene. At least six IBV genotypes together comprise 34 distinct viral lineages and a number of unassigned inter-lineage recombinants have been recognized worldwide according to a simple and repeatable phylogeny-based classification system that uses the complete nucleotide sequence of the S1 gene and an unambiguous and rationale lineage nomenclature for the assignment of IBVs (Valastro et al., 2016; Chen et al., 2017; Jiang et al., 2017).

There is also considerable variation in the virulence and tropism of IBVs, and in some cases the novel IBV strains emerged from point mutations, insertions, and/or deletions in the S1 gene. Another

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important mechanism that underlies the emergence of novel IBV strains is genomic recombination. In Australia, three recently isolated novel subgroup IBV strains were shown to be derived from recombination between subgroup 1 and 2 strains (Mardani et al., 2010). More recently, the complete genome analysis of newly emerged strains found multiple recombination events throughout the genome between wild-type viruses and vaccine strains (Quinteros et al., 2016). In the USA, the emergent virulent strain Ark DPI appears to have originated from recombination among four different IBV strains (Ammayappan et al., 2008). In addition, the emergence of new strains in the USA has been reported to have arisen from recombination events between the Massachusetts (Mass), Connecticut (Conn), and Holte strains as well as field strains (Thor et al., 2011). Recently in Europe, it has been shown that emergent viruses, such as the XDN-like virus in Spain and Italy (Moreno et al., 2016), and the γCoV/Ck/Italy/I2022/13 virus in Italy (Franzo et al., 2015), may have arisen from recombination events.

There have been several episodes of infectious bronchitis (IB) in Chinese chicken flocks, and the genotypes/serotypes of IBVs were previously classified based mainly on the nucleotide sequences of genes encoding the S1 subunit of the spike protein (Han et al., 2011), and in some cases based on cross virus-neutralization (Gao et al., 2016; Chen et al., 2017) in China. Since 1995, the predominant IBV type in China has been LX4 (also known as QX-like viruses), but molecular studies have shown that new types and variants are emerging continually (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). It has been suggested that the emergent IBV strains in China may have different origins. It is considered that some of the IBV types circulating in China, such as Mass and 793/B, are the most widely distributed types worldwide and they may have infected Chinese chickens from an exogenous source, probably due to the use of live vaccines (Chen et al., 2015; Han et al., 2017). By contrast, some IBV types, such as LX4 and ck/CH/LDL/97I (Q1-like), are believed to have originated in China and spread to other regions of the world (Valastro et al., 2016). The remaining IBV types, such as ck/CH/LSC/99I (Liu et al., 2006a,b), nrTW I (Xu et al., 2016), and GI-28 (Chen et al., 2017), are considered to be indigenous to China. The origins of most of the IBV types in China are still unknown, although it has been shown that some of these types have arisen from recombination events (Chen et al., 2015; Xu et al., 2016; Chen et al., 2017). The results of many of these previous studies are based on analyses of the available S1 gene sequences, but it is impossible to fully understand the origins and evolutionary processes related to these emerging viruses by only analyzing this small part of the genome.

In this study, we isolated and identified an nrTW I type IBV from an H120 and 4/91 vaccinated chicken flock with respiratory signs of IB. We sequenced the complete genome and compared it with other IBV sequences available in GenBank, and we then subjected these sequences to phylogenetic, molecular, and recombination analyses. We also investigated the antigenicity, pathogenicity, replication, and shedding of the nrTW I type IBV in chickens.

2. Materials and methods

2.1. Clinical samples, virus isolation, and viral stock preparation

Trachea samples were collected from five suspected IBV-infected broiler breeders and they were submitted to our laboratory in 2016 for routine diagnosis. Chickens in this flock had been vaccinated against IBV using the live attenuated H120 vaccine at 7 days of age, and the chickens were then boosted at 25 and 60 days with the live attenuated 4/91 and H120 vaccines, respectively. The birds were also vaccinated with a bivalent, inactivated vaccine, Newcastle disease virus La Sota/IBV M41, at 120 days. Some of the birds exhibited respiratory signs of IB at 150 days, which was accompanied by decreased egg production and abnormal shell quality in the breeding hens, and the clinical signs disappeared approximately 20 days later. The morbidity was approximately 5% and only a few birds died during this outbreak. Gross lesions were mainly associated with tracheitis and proventriculitis, and hepatitis and hydropericardium were observed in some chickens. Kidney lesions were not observed in the chickens examined.

To isolate the virus, the tracheal samples from the dead chickens were pooled and inoculated into 9-day-old specific pathogen-free (SPF) embryonated chicken eggs via the allantoic sac route, as described previously (Liu and Kong, 2004). The virus recovered from the third passage was named γCoV/ck/China/I1101/16 (I1101/16) (Ducatez and The European Unit COST Action FA1207, 2016) and it was used in the following experiments. The virus stock was prepared after an additional passage by inoculating the allantoic cavity of 9-day-old embryonated SPF chicken eggs. After incubation for 48 h, the allantoic fluid was collected aseptically and clarified by centrifugation at 2500 × *g* for 20 min, as described previously (Liu and Kong, 2004).

2.2. Eggs and chicks

Fertile White Leghorn SPF chicken eggs and White Leghorn SPF chicks were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, China. The birds were kept in isolation units with negative pressure throughout the experiment, and water and feed were provided *ad libitum*.

2.3. Viral RNA extraction, reverse transcription–polymerase chain reaction (RT-PCR) amplification, and complete genome sequencing

The complete genomes of the I1101/16 isolate and the ck/CH/LSC/99I strain were sequenced in this study. IBV strain ck/CH/LSC99I was isolated from preventriculus of a layer hen in Sichuan province in China in 1999 (Liu et al., 2006a,b). Genomic RNA was extracted from virus-inoculated allantoic fluids using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis and subsequent PCR were performed using a PrimeScript™ One-Step RT-PCR Kit ver. 2 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The complete genomes of the I1101/16 isolate and ck/CH/LSC/99I strain were amplified with primers used for amplifying the complete genomes of other Chinese IBV strains (Liu et al., 2013). The PCR profiles comprised initial denaturation for 5 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 50–60 °C (depending on the primer set) for 30 s, and 72 °C for 2 min. A 3′/5′ rapid amplification of cDNA ends kit (Takara Bio Inc.) was used to determine the 3′ and 5′ ends of the viral genomes according to the manufacturer's instructions (Liu et al., 2013).

The amplified products were sequenced directly or cloned into the pMD18-T vector (Takara Bio Inc.) according to the manufacturer's instructions. Each fragment of the viral genome was sequenced at least three times. The complete genomic sequences of the I1101/16 isolate and the ck/CH/LSC/99I strain were mapped using the genome sequences of the Beaudette and ck/CH/LJL/140901 IBV strains, respectively, in order to determine a consensus sequence.

2.4. Comparison and analysis of the S1 subunit of the spike and spike protein genes

The region coding for the S1 subunit of the spike protein is used for genotyping and classification purposes. Comparisons with published sequences were first performed using the nucleotide BLAST search tool in GenBank with the S1 gene nucleotide sequence from the I1101/16 isolate. The S1 nucleotide sequences from 70 IBV reference strains were downloaded from GenBank, where most of these IBVs were isolated in China and they represented the previously reported types (Valastro et al., 2016; Chen et al., 2017). Phylogenetic analyses were conducted