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Altered pathogenicity of a tl/CH/LDT3/03 genotype infectious bronchitis coronavirus due to natural recombination in the 5′- 17 kb region of the genome

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ABSTRACT

An infectious bronchitis coronavirus, designated as ck/CH/LGX/130530, was isolated from an IBV strain H120-vaccinated chicken in this study. Analysis of the S1 gene showed that isolate ck/CH/LGX/130530 was a tl/CH/LDT3/03-like virus, with a nucleotide sequence similarity of 99%. However, a complete genomic sequence analysis showed that ck/CH/LGX/130530 was more closely related to a Massachusetts type strain (95% similarity to strain H120) than to the tl/CH/LDT3/03 strain (86%), suggesting that recombination might have occurred during the origin of the virus. A SimPlot analysis of the complete genomic sequence confirmed this hypothesis, and it showed that isolate ck/CH/LGX/130530 emerged from a recombination event between parental IBV H120 strain and pathogenic tl/CH/LDT3/03-like virus. The results obtained from the pairwise comparison and nucleotide similarity showed that the recombination breakpoint was located in the *nsp14* gene at nucleotides 17055–17083. In line with the high S1 gene sequence similarity, the ck/CH/LGX/130530 isolate was serotypically close to that of the tl/CH/LDT3/03 strain (73% antigenic relatedness). Furthermore, vaccination with the LDT3-A vaccine, which was derived from the tl/CH/LDT3/03 strain by serial passaging in chicken eggs, provided good protection against challenge with the tl/CH/LDT3/03 strain, in contrast to the poor protection offered with the H120 vaccine. Interestingly, isolate ck/CH/LGX/130530 exhibited low pathogenicity toward specific-pathogen-free chickens compared with the nephropathogenic tl/CH/LDT3/03 strain, which was likely due to natural recombination in the 5′ 17-kb region of the genome. Our results also indicate that the replicase gene of IBV isolate ck/CH/LGX/130530 is associated with viral pathogenicity.

1. Introduction

Infectious bronchitis virus (IBV), a member of the *Gammacoronaviridae*, order *Nidovirales*, is an important pathogen that infects domestic chickens of all ages, causing an acute, highly contagious respiratory disease (Cavanagh and Gelb Jr., 2008). The IBV genome contains a single positive-strand RNA molecule, which is about 27.6 kb long and which has a cap at its 5′ end and a poly (A) tail at its 3′ end (Boursnell et al., 1987). The genome of IBV comprises ten open reading frames (ORFs), and the first 20 kb of the genome is made up of ORF1, which is a replicase gene. The replicase has two ORFs, 1a and 1b, and 1b is produced as a 1ab polyprotein by a −1 ribosomal frame-shifting mechanism. ORF1 encodes 15 non-structural proteins associated with RNA replication and transcription. The IBV genome encodes four major structural proteins: the spike (S) glycoprotein, the small envelope (E) protein, the membrane (M) glycoprotein, and the nucleocapsid (N) protein (Cavanagh and Gelb, 2008). In addition, IBV has other genes that encode for proteins interspersed among structural genes, namely 3a, 3b, 5a, and 5b (Boursnell et al., 1987).

Numerous serotypes have been described for IBV thus far, as frequent point mutations occur in the S1 domain of the S gene of the virus. Another important feature of IBV, as well as other coronaviruses, is the high rate of homologous and non-homologous RNA–RNA recombination that has been demonstrated to occur among selected and unselected markers during the course of infection (Masters, 2006). RNA recombination in coronaviruses is thought to result from a copy-choice mechanism (Kirkegaard

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and Baltimore, 1986). In this scheme, the viral polymerase, with its nascent RNA strand intact, detaches from one template and resumes elongation at the identical position, or a similar position, on another template. Although strong selective pressures are able to create the appearance of local clustering of recombinational hot spots (Banner et al., 1990), the sites of recombination are considered to be random (Banner and Lai, 1991). Because homologous recombination can occur between genomic and subgenomic RNAs, with the latter providing a source of donor and acceptor templates that become more numerous as a function of proximity to the 3′ end of the genome, the rate of recombination increases from the 5′ to 3′ end of the coronavirus genome, (Masters, 2006).

Accumulating evidence suggests that recombination is an inherent component in IBV evolution, and it is believed that recombination events can occur in the field under the following conditions: when there are extremely large numbers of chickens, especially when kept at high density; when the virus is easily spread; and when there is a co-circulation of serotypes, including proof of co-infection with more than one serotype in a given flock (Cavanagh, 2007). The circumstantial evidence for recombination, derived from gene sequence comparisons, has shown that the recombination events had occurred a very long time ago. However, it was shown that recombination in the S1 domain of the S protein of the IBV genome is a mechanism that leads to the emergence of new IBV strains, and it could be responsible for changes in viral pathogenicity and replication in a single generation (Hewson et al., 2014). In general, it is difficult to ascertain what effect recombination has on the serotype, pathogenicity, virulence, and tissue tropism of recombinant strains using only gene sequence analyses. Serotyping and animal studies, paired with differential analyses of genes, genomic sequencing, and phylogenetic analyses, will be essential to provide insights into the altered characteristics of recombinant IBV strains and their effect on poultry health.

2. Materials and methods

2.1. Epidemiological background and virus isolation

An IBV strain, designated as ck/CH/LGX/130530, was isolated from tracheal swabs of diseased broilers suspected of having an IBV infection in Guangxi province, China, in 2013. The flock investigated in this study contained about 15,000 broilers. One-day-old chickens were vaccinated against IBV with the live attenuated H120 vaccine, and 15-day-old chickens boosted with Ma5 live vaccines. Some of the chickens showed early clinical signs, including listlessness, huddling, and ruffled feathers, when they were 20 days old. On day 25, some of the diseased chickens began to die, and gross examinations showed mild to severe tracheitis, nephritis, and proventriculitis. The morbidity rate was about 7%, and the mortality rate was 3%. The clinical signs of the chickens tended to disappear gradually after about day 35.

Tracheal swabs were collected from 30 chickens in the diseased flock on day 23 and centrifuged at $6000 \times g$ for 10 min. The supernatants were pooled and inoculated into five 9-day-old embryonated specific-pathogen-free (SPF) eggs via the allantoic cavity (0.2 ml per egg). The eggs were candled daily, and embryos deaths that occurred within 24 h of inoculation were considered to be non-specific. Allantoic fluids were collected at 96 h post-inoculation, pooled together, and clarified by centrifugation at $3000 \times g$ for 5 min. The clear supernatant was used for further passaging. The samples were blind passaged four times, and characteristic IBV lesions, such as dwarfing, stunting, or curling of embryos, were observed at the fourth passage level. Allantoic fluids of the inoculated embryos were collected for reverse transcription-PCR (RT-PCR) amplification as previously described (Liu and Kong, 2004).

In addition, a pathogenic IBV strain, tl/CH/LDT3/03, and two vaccine strains, H120 and LDT3-A; the LDT3 was derived from the tl/CH/LDT3/03 strain by serial passaging in chicken eggs, were used in the virus cross-neutralization and vaccination-challenge tests in this study. The pathogenic strain tl/CH/LDT3/03 and vaccine strain LDT3-A were also used for complete genomic sequencing. Embryo-propagated viral stocks of these viruses were produced by inoculating the virus into embryonated SPF chicken eggs via the allantoic cavity, and the infectious allantoic fluid was collected 48 h post-inoculation as previously described (Liu and Kong, 2004). The titers of the four viruses, ck/CH/LGX/130530, tl/CH/LDT3/03, H120, and LDT3-A, were determined by inoculating 10-fold dilutions into groups of five 10-day-old embryonated chicken eggs. The median embryo infectious dose (EID_{50}) was calculated using the method of Reed and Muench (1938).

All experiments were conducted using standard procedures with the formal approval of the Ethical and Animal Welfare Committee of Harbin Veterinary Research Institute, China.

2.2. RNA extraction, gene amplification, and sequencing

Genomic RNA was extracted from virus-infected allantoic fluid with the RNAiso Plus kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions and stored at −80 °C until further use. Twenty overlapping primers (Zhang et al., 2015) were used to amplify the genomes of the isolate ck/CH/LGX/130530, strain tl/CH/LDT3/03 and the LDT3-A vaccine strain. All gene fragments were amplified using the RT-PCR kit (TaKaRa) according to the manufacturer's instructions. The 3′/5′-termini of the three IBV strains were determined as previously described (Zhang et al., 2015) using the 3′/5′ RACE kit (TaKaRa) according to the manufacturer's instructions. The RT-PCR products were sequenced directly and/or cloned into the pMD18-T vector (TaKaRa), and three independent clones were sequenced for each amplicon.

2.3. Sequence analysis

The assembly of contiguous sequences of ck/CH/LGX/130530, tl/CH/LDT3/03 and LDT3-A was performed with GeneDoc software (Ammayappan and Vakharia, 2009). Both the S1 gene and complete genomic sequences of IBV isolate ck/CH/LGX/130530 were used for BLAST searching of the National Center for Biotechnology Information (NCBI) database. The tl/CH/LDT3/03 and partridge/GD/S1/42003 strains, as well as the LDT3-A vaccine, were used as one group of parental viruses for sequence comparisons. In addition, Massachusetts type H120 and Mass 41 strains were selected and used as another group of parental viruses for sequence comparisons. Similarity and breakpoint analyses of the complete genomic sequence of ck/CH/LGX/130530 were aligned with those of tl/CH/LDT3/03, LDT3-A, partridge/GD/S1/42003, H120, and Mass 41, and the multiple alignment results were introduced into SimPlot version 3.5.1 to identify likely recombination breakpoints (Lole et al., 1999). SimPlot analyses were performed using a 1000 bp window with a 100 bp step. IBV strain Mass 41 was used as the query strain. To confirm regions in which recombination events occurred in the genome of the ck/CH/LGX/130530 isolate, pairwise comparisons of the complete genomic sequence of ck/CH/LGX/130530 were performed with those of tl/CH/LDT3/03, LDT3-A, H120, and M41, and the nucleotide similarities of the different corresponding gene fragments were calculated. In addition, pairwise comparisons of the sequence of the 3′ 7.0 kb region of ck/CH/LGX/130530 were performed with those of partridge/GD/S1/42003, tl/CH/LDT3/03, and LDT3-A to elucidate whether the tl/CH/LDT3/03-like sequence