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Identification and molecular characterization of a novel serotype infectious bronchitis virus (GI-28) in China



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ABSTRACT

Avian infectious bronchitis coronavirus (IBV) is a major poultry pathogen. A characteristic feature of IBV is the occurrence of many different strains belonging to different serotypes, which renders complete control of the disease by vaccination a challenging task due to the poor cross-protection between different serotypes. In this study, based on the results of S1 sequence analysis and virus cross-neutralization tests, IBV strain ck/CH/LGX/111119 was found to be genetically and antigenically different from other known IBV types, representing not only a novel genotype, but also a novel serotype (designated as GI-28). Viruses belonging to this novel serotype have been isolated from several regions in China in recent years, suggesting endemic circulation of the serotype in various geographic locations in China. Further studies by complete genomic analysis showed that strain ck/CH/LGX/111119 may have originated from recombination events involving LX4 genotype IBVs and an as-yet-unidentified IBV donating a S1 gene, or from the result of accumulation of mutations and selections, especially in the S1 gene, from a LX4 genotype virus. ck/CH/LGX/111119 is a nephropathogenic strain, although it had broader tissue tropism (respiratory, digestive, urinary, and reproductive tracts) among chickens challenged at one day old. Infection of the oviducts with ck/CH/LGX/111119 found in this study may have severe implications because the virus will likely induce the occurrence of false layers.

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1. Introduction

Coronaviruses, family Coronaviridae in the order Nidovirales, are generally responsible for mild enteric and respiratory infections in both humans and animals (Masters, 2006). They are now recognized as emerging viruses with a propensity to cross into new host species, as have been shown by previous and recent outbreaks of severe acute respiratory syndrome and Middle East respiratory syndrome (Coleman and Frieman, 2014). To date, coronaviruses were classified into four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The genus *Gammacoronavirus* is mostly composed of viruses isolated from birds. However, gammacoronaviruses have also been detected in the beluga whale (Mihindukulasuriya et al., 2008)

and bottlenose dolphin (Woo et al., 2014). The most economically important of the avian gammacoronaviruses are infectious bronchitis virus (IBV) and turkey coronaviruses. IBV, which was the first identified coronavirus and was isolated in the 1930s, causes highly contagious infectious bronchitis in domestic fowl, a respiratory, renal, and genital disease, which causes serious economic consequences worldwide (Cavanagh, 2007).

Coronaviruses are enveloped, positive-sense, and the largest known RNA viruses, having a genome length of approximately 30 kb. About two-thirds of the genome consists of two large overlapping open reading frames (ORFs), ORF1a and ORF1b, which are translated as the polyproteins pp1a and pp1ab, and then processed by virus-encoded proteinases into 15 or 16 non-structural proteins (Ziebuhr, 2005). The remaining one-third of the genome encodes the virus structural spike (S), membrane (M), enveloped (E), and nucleocapsid (N) proteins, as well as four non-structural accessory proteins (3a, 3b, 5a and 5b). The S glycoprotein can be cleaved into two subunits, S1 and S2, and contains the regions of both B- and T-cell epitopes that are important for virus neutralization and the reorganization of virus-infected cells

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(Reguera et al., 2012; Satoh et al., 2011). The S1 domain occupies the amino proximal half, which contains the receptor-binding domain, and the S2 domain occupies the other half, which contains elements involved in membrane fusion.

In contrast to species belonging to the alpha- and betacoronaviruses, which occur as only one or two different serotypes, there are many different serotypes/genotypes of the chicken coronavirus IBV (Wickramasinghe et al., 2014). Typing of IBV strains is based on the feature of the S protein, especially S1 domain (Cavanagh, 2007). The high mutation and recombination rate of IBV, especially in the S1 subunit of the S protein gene, has led to the emergence of new variants, particularly in Europe and North America, and more recently in intensive poultry farms in China. Among the IBVs, two very important genotypes, LX4 (QX-like) and ck/CH/LDL/971, are believed to have originated in China in mid-1995 and have since spread to other regions of the world (Ababneh et al., 2012; Marandino et al., 2015). More recently, another novel genotype, Guandong/Xindadi (XDN), which is also believed to have originated in China from independent recombination events between the LX4 and 793/B IBV genotypes, have emerged in Italy and Spain (Moreno et al., 2016). The continuous emergence of new IBV genotypes in China that later spread to other regions of the world has not only prompted the development of appropriate control programs aimed to mitigate the occurrence of the disease caused by those new IBV genotypes, but also emphasize the importance of continuous surveillance of IBV in chicken flocks in China. Meanwhile, extensive investigations, involving molecular characterization (especially the complete genome), antigenicity, and pathogenicity, are necessary to clarify the origin of the viruses and are significant for the control of diseases caused by new variants. In this study, a novel serotype (designated as GI-28) of IBV was found to be mainly circulated in south China in recent years and the complete genome, antigenicity, and pathogenicity were investigated to elucidate the origin and evolution of the novel serotype.

2. Materials and methods

2.1. Virus isolation

The IBV strain ck/CH/LGX/111119 was isolated from the proventriculus of a diseased 80-day-old yellow broiler that had been vaccinated against IBV with live attenuated H120 vaccine at 7 days old and boosted at 25 days old with live attenuated 4/91 vaccine. The bird showed obvious respiratory signs at 70 days old. In this flock, morbidity was about 15% and mortality was 3%. Gross lesions were mainly mild with tracheitis, proventriculitis, and nephritis in some chickens. The proventriculi of the dead chickens were enlarged and had pale or mottled pale serosa. Kidneys of the birds were enlarged and pale. Virus isolation was performed as described by Liu and Kong (2004). Briefly, the proventriculi were homogenized, clarified by centrifugation, filtered, and then inoculated into specific pathogen-free (SPF) eggs (Harbin Veterinary Research Institute, Harbin, China) on embryonation day 9 via the allantoic cavity. The isolate were purified and propagated by inoculating and passaging three times in the allantoic cavity of 9-day-old SPF embryonated chicken eggs until characteristic IBV lesions, such as dwarfing, stunting, or curling of embryos, were observed (Liu and Kong, 2004; Hewson et al., 2009).

2.2. RNA extraction and viral genome sequencing

The infectious allantoic fluid was clarified by centrifugation at 5000g for 10 min at 4°C. Total viral RNA was extracted from the supernatant using TRIzol reagent (Invitrogen Corporation,

Carlsbad, CA, USA), following the manufacturer's protocol, and then stored at -80°C for further use.

IBV-specific primers (Liu et al., 2013) spanning the entire viral genome were used to reverse transcribe and amplify the viral RNA contained in 2 µl of extracted total RNA using the PrimeScript™ One-Step RT-PCR Kit ver. 2 (Takara Bio Inc., Shiga, Japan) as described by the manufacturer. Briefly, a 25-µl reaction was set up on ice containing 2 µl of RNA, 1 µl of 10 µM forward, 1 µl of 10 µM reverse primer, 12.5 µl of 2 × 1 step buffer (as supplied by the manufacturer), 1 µl of PrimeScript 1 step Enzyme Mix, and water to a final volume of 25 µl. The reaction was incubated at 50°C for 30 min to allow cDNA synthesis, and then increased to 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 50°C–60°C (depending on the primer set) for 30 s, and 72°C for 2 min. The reaction underwent a final extension phase at 72°C for 10 min and was then held at 4°C. The 3' and 5' ends of the viral genomes were confirmed by rapid amplification of cDNA ends using a commercial kit (Takara Bio, Inc.) as described by Liu et al. (2013). For each amplicon, 5 µl of PCR products were separated on a 1% agarose/Tris/borate/ethylenediaminetetraacetic acid gel to confirm the PCR product size and to estimate the amount of DNA by comparison with standards. The PCR products were sequenced directly and/or cloned into a pMD 18-T vector (Takara Bio Inc.) following the manufacturer's instructions. For each amplicon, three to five clones were sequenced.

2.3. Genotyping, sequence comparison, and recombination analysis

The S1 gene of strain ck/CH/LGX/111119 was used to search the GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the BLASTN program for IBV sequences. Six sequences sharing more than 95% nucleotide identity with ck/CH/LGX/111119 were selected for construction of a phylogenetic tree for sequence comparison. In addition, the S1 gene sequences of 93 IBV reference strains with different genotypes were also selected for comparison in this study (Fig. 1) (Valastro et al., 2016). The phylogenetic tree was constructed using a neighbor-joining algorithm with 1000 bootstraps based on an alignment generated using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. (Tamura et al., 2007). The S1 nucleotide and amino acid sequences of selected viruses (based on the phylogenetic tree results) were also aligned and compared with those of strain ck/CH/LGX/111119 using Lasergene software (DNASTar, Inc., Madison, WI, USA). Percent similarities were calculated to determine distances between nucleic acid and amino acid pairs.

To determine a consensus sequence for the complete genome of strain ck/CH/LGX/111119, the sequence contigs were combined, manually edited, and assembled using Lasergene software. The genomic sequences between ck/CH/LGX/111119 and other reference IBVs were analyzed using Lasergene software to map each of the genes in the genome of strain ck/CH/LGX/111119. The sequence of the complete genome of strain ck/CH/LGX/111119 was submitted to the GenBank database under the accession number KX640829.

To identify the recombination events, the BLASTN program was used to search the GenBank database for IBV sequences that were homologous to strain ck/CH/LGX/111119. SimPlot analyses were performed using a 1000-bp window with a 100-bp step. IBV strain H120 was used as the query strain.

2.4. Virus cross-neutralization test

Six IBV strains, including ck/CH/LGX/111119 in this study and five other strains representing five serotypes (4/91 for 793/B serotype, H120 for Massachusetts serotype, ck/CH/LDL/140520 for TW I serotype, ck/CH/LDL/971 for ck/CH/LDL/971 serotype, and ck/CH/LDL/091022 for LX4 serotype) were subjected to one additional

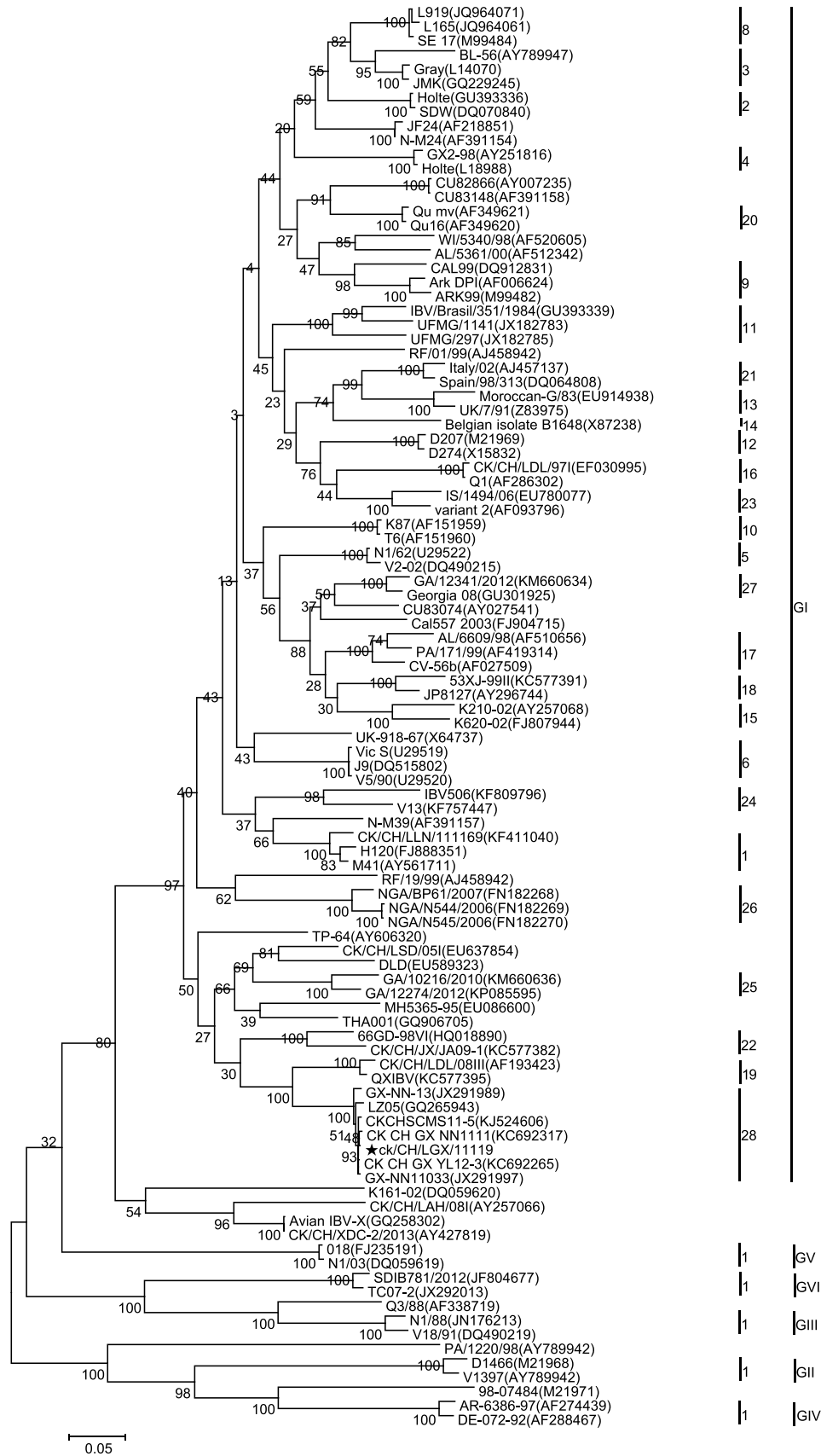


Fig. 1. Phylogenetic trees constructed with the nucleotide sequence alignments of the S1 glycoprotein genes using the nearest neighbor-joining method with 1000 bootstrap calculations (Valastro et al., 2016). The GenBank accession numbers of the IBV strains are shown in parentheses. The strain ck/CH/LGX/11119 is indicated by a ★. All the IBV strains were clustered into 6 genotypes (GI–GVI) and a number of inter-lineage recombinants. Ninety-three IBV reference strains in GI were further grouped into 27 distinct viral lineages (GI-1–GI-27) (Valastro et al., 2016). Our ck/CH/LGX/11119 strain was clustered into a novel lineage in GI with other 6 IBV reference strains and designated as GI-28.

passage in embryonated chicken eggs for virus stock preparation and cross-neutralization tests. The virus titers were determined in 9-day-old SPF chicken embryos by the allantoic route of inoculation as described by Chen et al. (2015). In addition to embryo microscopic changes, IBV RNA in allantoic fluid was detected by RT-PCR as described by Chen et al. (2015) to determine virus replication and subsequently calculate the virus titer. The median embryo infectious dose (EID₅₀) was calculated using the method of Reed and Muench (1938). The antisera used for this test included anti-4/91, anti-H120, anti-ck/CH/LDL/140520, anti-ck/CH/LDL/971, anti-ck/CH/LDL/091022 (Gao et al., 2016), and anti-ck/CH/LGX/111119, which were prepared as described by Gao et al. (2016).

For virus neutralization, the β VN method with constant virus and diluted serum was employed in SPF chicken embryos for serotyping. Briefly, sera were serially diluted two-fold and mixed with 100 EID₅₀ of the IBV strains. After incubation for 1 h at 37 °C, each of the virus-serum mixtures was inoculated into the allantoic cavity of five SPF chicken embryos, which were observed for 7 days. Similarly, IBV RNA in allantoic fluid was detected by RT-PCR and used to determine virus replication and subsequently calculate the end-point titer of serum. The end-point titer of each serum sample was calculated using the method of Reed and Muench (1938).

2.5. Pathogenicity test

Thirty 1-day-old SPF white leghorn layer chickens were separated into two groups of 15 chickens per group, which were housed in separate isolation units. Birds in group 1 were challenged with the ck/CH/LGX/111119 strain at 1-day-old via the intranasal and ocular routes with 10⁶ EID₅₀ in 0.1 ml of diluent per bird. Birds in group 2 were not challenged and served as negative controls. The chicks were examined daily for clinical signs of infection (Gao et al., 2016). Morbidity and mortality were recorded daily. Five chickens in each group were humanly killed using carbon dioxide/oxygen. The dead chickens were carefully examined, especially for lesions in the trachea, kidneys, and proventriculi. Then, tissues of the tracheal, lungs, kidneys, and cecal tonsils were collected, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 4 μ m, and mounted on glass slides, which were stained with hematoxylin and

eosin. Immunohistochemical (IHC) analysis was performed to detect the IBV antigen using monoclonal antibody 6D10 against the N protein as described by Han et al. (2016) and Gao et al. (2016). Blood samples were collected on day 4 from 10 birds and on days 8, 12, 16, 20, and 24 post-challenge from all birds to confirm the presence of antibodies against IBV using a commercial enzyme-linked immunosorbent assay kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) in accordance with the manufacturer's instructions. The percentage of seropositive birds for each group in this study was calculated. All surviving birds were killed humanely using carbon dioxide/oxygen at 3 months of age, which was followed by exsanguination. Post-mortem examinations were performed with special attention to abnormalities in the oviducts and kidneys. The kidney and oviduct samples were collected to detect the presence of IBV by IHC analysis.

3. Results

3.1. IBV GI-28 genotype has been circulating in chicken flocks in China in recent years

Genotyping based on the phylogenetic analysis of the S1 gene from our ck/CH/LGX/111119 and 99 reference IBV strains assigned the viruses into different clusters (Fig. 1). The 93 IBV reference strains were clustered into 6 genotypes that together comprise 32 distinct viral lineages (GI-1– GI-27; GII– GVI) and a number of inter-lineage recombinants (Valastro et al., 2016). The isolate ck/CH/LGX/111119 in this study was clustered into a novel lineage in genotype I (GI) with 6 IBV strains isolated in China and clearly set apart in the phylogenetic tree from the 93 reference strains; hence, we designated the novel genotype as GI-28. Viruses of the GI-28 genotype possessed more than 97.7% and 96.0% nucleotide and amino acid identities, respectively, between each other in this study, supporting the classification of these viruses as a novel genotype.

One IBV strain lie together with the QXIBV strain, and have been described previously as LX4 genotype or QX-like viruses (Liu and Kong, 2004). Comparatively, the LX4 genotype (QX-like) viruses are closely related to the GI-28 genotype, sharing approximately 87.1% and 87.3% nucleotide and amino acid identities, respectively, with the S1 protein of the GI-28 genotype viruses selected in this

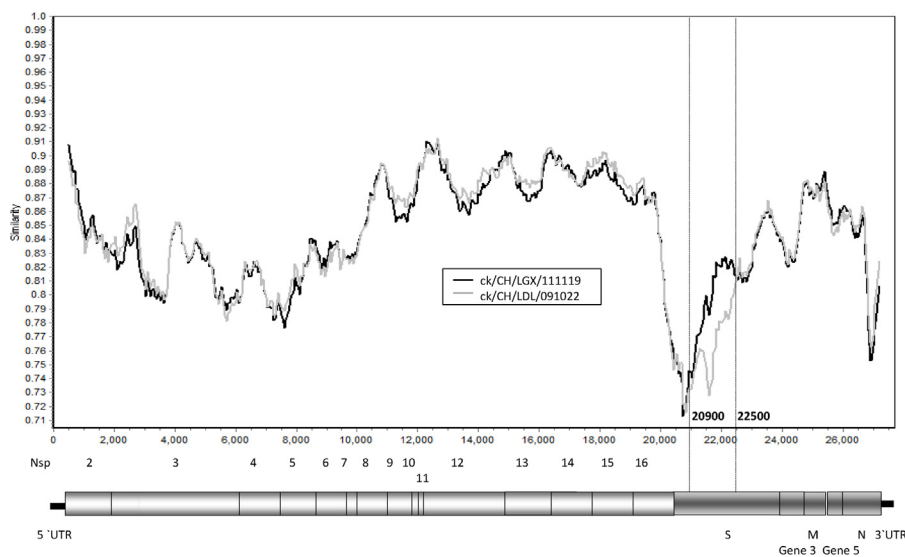


Fig. 2. SimPlot analysis of the complete genomic sequences of strains ck/CH/LGX/111119 and ck/CH/LDL/091022. Analysis was performed using SimPlot software version 3.5.1 to identify potential recombination breakpoints (Chen et al., 2015). A 1000-bp window with a 100-bp step was used. Strain H120 was used as the query strain.

study. The Massachusetts genotype H120 had less than 80% nucleotide and amino acid identities with the S1 protein of GI-28 genotype viruses. The TW I genotype, which has been described previously (Xu et al., 2007). The S1 protein of genotypes GI-28 and TW I shared approximately 80% nucleotide and amino acid identities. The strains ck/CH/LDL/971 and Q1 formed a unique cluster that shared the lowest identities with the GI-28 genotype (nearly 75%). The 793/B genotype shared about 78% and 76% nucleotide and amino acid identities, respectively, with the S1 protein of the GI-28 genotype.

3.2. Genome characterization of GI-28 genotype

The genome of IBV strain ck/CH/LGX/111119 is 27,667 nucleotides in length, excluding the 3' poly(A) tail, and shows typical IBV organization. The 5' end of the viral genome contains a 525-nt 5' untranslated region (UTR) followed by a 19889-nt replicase gene encoding for two large polyproteins, pp1a and pp1ab, which occupied about two-thirds of the viral genome. Four structural and accessory proteins were downstream of the replicase gene followed by a 504-nt UTR at the 3' end of the genome.

Results of a BLASTN search showed that not only the S1 gene, but the complete genome of the ck/CH/LGX/111119 strain, showed the closest genetic relatedness with LX4 genotype strains. Hence, the LX4 genotype strain ck/CH/LDL/091022 was used for SimPlot analysis with strain ck/CH/LGX/111119. As illustrated in Fig. 2, a fragment from approximately nt position 20,900 to 22,500 in the genome of strain ck/CH/LGX/111119 was obviously different from that of strain ck/CH/LDL/091022. This approximate 1600-nt fragment occupied most of the S1 gene and small parts of sequence at the 5' end of the S2 gene. The remaining regions in the genome were very similar, although some regions showed slight diversities between the two strains, implicating the involvement of LX4 genotype IBVs in the origin of ck/CH/LGX/111119-like strains.

3.3. GI-28 genotype represents a novel serotype

The results of the cross-neutralization tests using the IBV strain ck/CH/LGX/111119 and antisera against the five IBV strains, which represented the Massachusetts, 793/B, LX4, ck/CH/LDL/971, and TW I serotypes, showed that the strains belonged to different serotypes (Table 1). In line with the genotyping results, strain ck/CH/LGX/111119 was antigenically distinct from the Massachusetts, 793/B, ck/CH/LDL/971, and TW I serotypes. Comparatively, the cross-neutralization results showed that strain ck/CH/LGX/111119 was antigenically close to the LX4 genotype strain ck/CH/LDL/091022 (Table 1) with an *r* value of 0.306. These results demonstrated that strain ck/CH/LGX/111119 represents a new serotype (also designated as GI-28) that is antigenically distinct from other IBV serotypes.

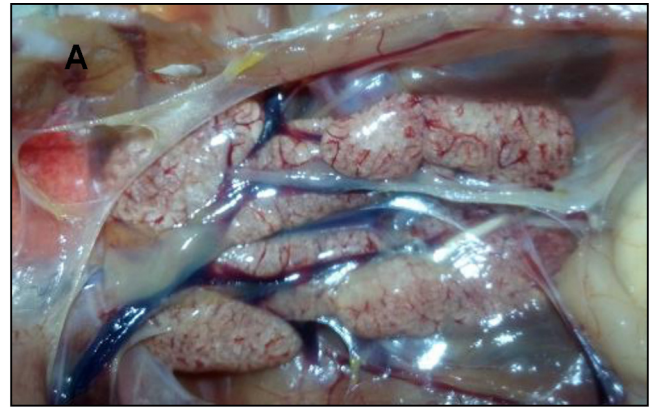


Fig. 3. Renal lesions of the kidney (A) and cystic dilation of the oviduct (B) in SPF chickens caused by inoculation with the GI-28 type ck/CH/LGX/111119 strain. Kidneys are swollen, tubules and ureters are distended, and uric acid crystals are present.

3.4. GI-28 serotype is highly pathogenic to SPF chicken

All of the chickens exhibited mild respiratory signs at 2–4 days post-challenge (dpc) with the IBV strain ck/CH/LGX/111119, and three of them died at 5, 7, and 10 dpc, respectively. The most remarkable lesions were confined in the kidneys of the five chickens killed at 5 days pdc and the three dead chickens. The affected kidneys were swollen and pale with tubules and ureters distended with urates (Fig. 3A). The most interesting observation was the dilatation and serous fluid accumulation in the oviducts of two of the three layer hens that survived 90 dpc (Fig. 3B). No clinical signs were observed and no birds died in the control group during the experimental period. No lesions were found in the kidneys or oviducts of the birds in the control group at the end of the experiment.

Viral antigens were detected by IHC analysis in the kidneys, secondary bronchi (Fig. 4A and B), and enterocytes of the cecal

Table 1

Results of reciprocal β virus neutralization tests using ck/CH/LDL/14520 and other IBV strains (serum dilution using a constant amount of virus).

Virus	Serum					
	ck/CH/LGX/111119	ck/CH/LDL/971	4/91	ck/CH/LDL/091022	H120	ck/CH/LDL/140520
ck/CH/LGX/111119	36.8 ^a	<2	<2	6.9	<2	<2
ck/CH/LDL/971	6.5	79.9	–	–	–	–
4/91	6.7	–	675.6	–	–	–
ck/CH/LDL/091022	288	–	–	576	–	–
H120	<2	–	–	–	205	–
ck/CH/LDL/140520	11.3	–	–	–	–	910.2

–, Not tested.

^a Reciprocal titer.

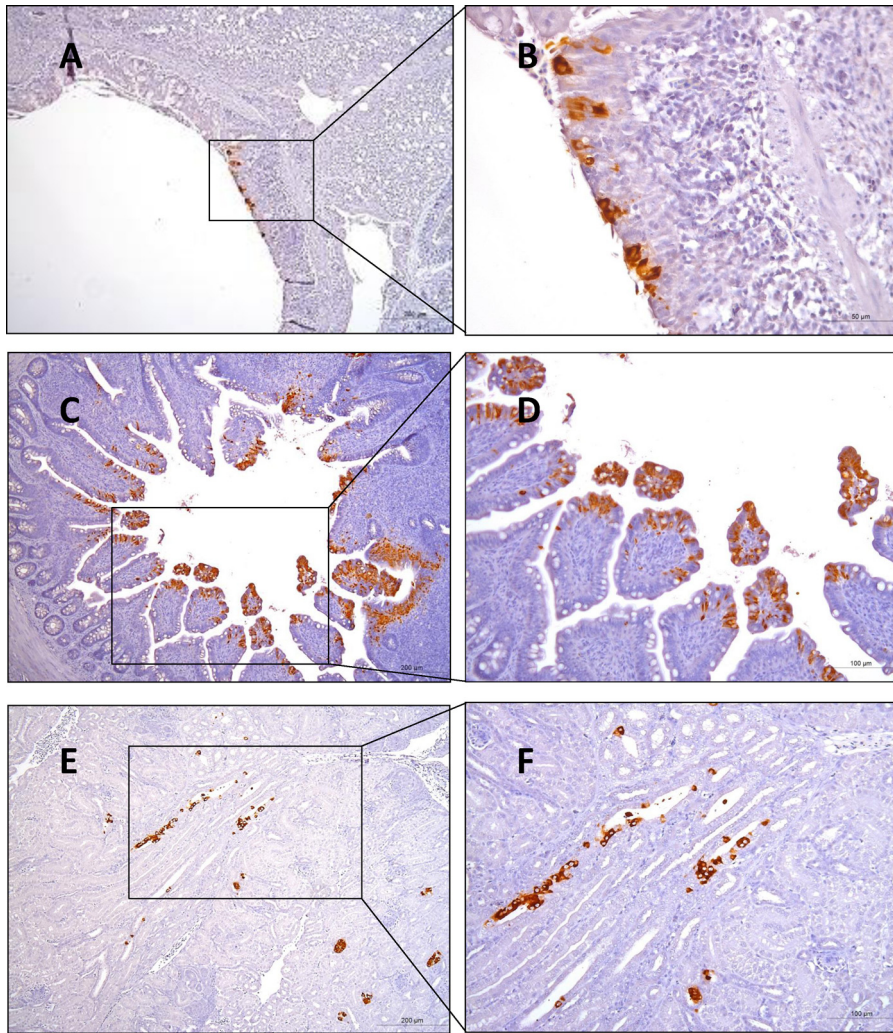


Fig. 4. Detection of IBV antigen by immunohistochemical analysis in the bronchi (A and B), enterocytes of the cecal tonsils (C and D), and kidneys (E and F) of dead chickens infected with the IBV GI-28 type ck/CH/LGX/111119 strain.

tonsils of the five chickens infected with strain ck/CH/LGX/111119 at 5 dpc (Fig. 4C and D). Obvious lesions (nephritis) were found in the kidneys of the five chickens killed at 5 days pdc and the three dead chickens, and IHC-positive cells were observed in the kidneys of the five chickens killed at 5 days pdc with IBV strain ck/CH/LGX/111119 as nephropathogenic strains (Fig. 4E and F). Most of the birds seroconverted by 8 dpc and all seroconverted by 12 dpc (Table 2). There was no seroconversion in any chicken in the negative control group.

4. Discussion

IBV has progressively emerged as the cause of moderate to severe disease in chickens, with different variants/genotypes/serotypes being detected in recent years in China. The IBV isolate ck/CH/LGX/111119, investigated in the present study, displayed distinct molecular features with respect to other genotypes and was clearly set apart from those that are not only used as vaccines in China like Massachusetts and 793/B, but also from Chinese variants of IBV, such as genotypes LX4, ck/CH/LDL/971, and TW I.

Table 2
Pathogenicity test results of SPF chickens infected with strain ck/CH/LGX/111119.

Groups	Morbidity	Mortality	Lesions ^a		Cystic oviducts ^c	Antibody response ^b						
			Nephritis			4	8	12	16	20	24	
			Dead	Survival								
ck/CH/LGX/111119	15/15	3/10	3/3	0/7	2/3	0/10	6/8	7/7	7/7	7/7	7/7	7/7
Negative control	0/15	0/10	–	0/10	–	0/10	0/10	0/10	0/10	0/10	0/10	0/10

^a The post-mortem examinations were only conducted for chickens that died from challenge with ck/CH/LGX/111119 strain, as well as for the chickens that survived 90 days post-challenge. All of the dead chickens, but none of the surviving chickens, had nephritis.

^b Antibody responses against IBV were examined from 4 to 24 days post-challenge.

^c Only three of the surviving chickens were hens, which were examined for oviduct abnormalities.

The closest relative genotype was IBV LX4, the most predominant variant in China (Liu and Kong, 2004), which shared approximately 87% nucleotide and amino acid identities with isolate ck/CH/LGX/11119. There are several publications of the IBV genotype that included ck/CH/LGX/11119, but unfortunately these reports do not agree on genotype designations, such as A2 by Ji et al. (2011) and CH III by Luo et al. (2012) and Feng et al. (2014). Also, the virus genotype designations were inconsistent even in reports by the same research group (Ji et al., 2011; Feng et al., 2014). The CH III and A2 genotypes have previously been used to classify other IBV genotypes in China (Liu et al., 2006; Xu et al., 2007). Herein, by comparison with the results from Valastro et al. (2016), the IBV reported in this study was designated as the GI-28 genotype.

Comparison of the S1 genes of the GI-28 genotype with those of other genotypes selected in this study revealed not more than 87.5% nucleotide and amino acid identities, which could explain the distinct antigenic relationship of ck/CH/LGX/11119, as compared with other serotypes (Cavanagh, 2007). Analysis of complete genomic sequence of the ck/CH/LGX/11119 revealed that the virus exhibited high identity in the entire genome with LX4 genotype IBVs with the exception of the S1 protein gene, which is markedly diverse. We proposed that the emergence of GI-28 genotype viruses in chicken flocks in China resulted from recombination events involving LX4 genotype IBVs and an as-yet-unidentified IBV donating a S1 gene that encoded a protein of low amino acid identity to those of other IBVs (approximately 87.3%). This was very similar to that of turkey coronavirus, which was thought to have arose through recombination of the S gene (Jackwood et al., 2010). Alternatively, the GI-28 serotype might have originated from the accumulation of mutations and selection, especially in the S1 gene, because S1 domain showed the high sequence diversity in IBV (Wickramasinghe et al., 2014). If this is the case, strain ck/CH/LGX/11119 might have undergone extensive divergent evolution and acquired the abilities to infect and spread among chicken flocks. The higher sequence similarity in other regions in the genome outside of the S1 gene suggest a common origin of these sequences and could explain the closer antigenicity between ck/CH/LGX/11119 and ck/CH/LDL/091022, as compared with those of other serotypes, because in addition to the five conformation-dependent neutralizing antigenic sites mapped on S1, another immunodominant region was mapped in the N-terminal region of S2 (Wickramasinghe et al., 2014) and some antigenic epitopes that have roles in the protection have also been identified on N proteins of IBV (Ignjatovic and Sapats, 2005).

In this study, mild respiratory signs developed in the chicks at 2–4 dpc and severe nephritis present in the dead chickens was caused by infection with the isolate ck/CH/LGX/11119 and no obvious lesions were observed in the proventriculus. This is in contrast to the observation in the field cases, where obvious respiratory signs and proventriculitis were observed. There are at least two possible reasons for this discrepancy: First, IBV strain ck/CH/LGX/11119 may not be the only causative pathogen responsible for the occurrence of the outbreak, and secondary infections caused by other microorganisms enhance the severity and contribute to the disease development. Second, it is also possible that different genetics of the chicks in the field cases and of the chicks experimentally infected are responsible for the difference in tropism of the virus; it is believed that genetics of chickens play an important role in differences in IBV susceptibility and tropism (Ignjatovic et al., 2003). It is believed that IBV mainly infects the respiratory and urogenital tracts, but it has long been known that IBV can be isolated from digestive organs, such as the proventriculus, intestines, and cloaca, and persists in the cecal tonsils (Ambali and Jones, 1990; Ambali, 1992; Montgomery et al., 1997). Similarly, the results of this study showed that obvious positive immunohistochemical reactions developed not only in the cytoplasm of the

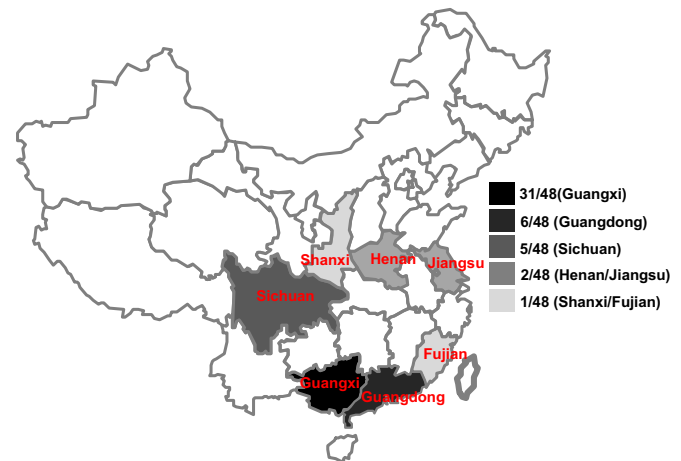


Fig. 5. The grey regions of the map show seven Chinese provinces, where the 48 GI-28 type viruses were isolated. The difference in gray color is related to the numbers of viruses (shown on the right).

tubular epithelial cells and in the mucous membrane of the ureters and collecting ducts in the kidney, and in the epithelial cells of the tracheal mucosa of the secondary bronchi at 5 dpc, but the viral antigens were also detected in the cecal tonsils. This result suggests a broad tissue tropism of strain ck/CH/LGX/11119, similar as that of its deduced parental LX4 genotype viruses (Benyeda et al., 2009; de Wit et al., 2011; Mork et al., 2014).

The clinical manifestations of IBV in the oviduct are of high economic importance in the poultry industry because an infection of the oviduct may have severe implications comprising a drop in egg production, poor egg quality, and the occurrence of so-called “false layers.” It has been reported that IBV genotypes LX4 (Benyeda et al., 2009; de Wit et al., 2011), Massachusetts serotype H52 (Duff et al., 1971), IS/885/00-like virus isolated in the middle-eastern region (Awad et al., 2016), and nrTW I serotype viruses isolated in China (Gao et al., 2016) related to cystic oviducts in layer flocks and induce false layers. An interesting observation in this study was that the lesions of cystic oviducts were observed in the surviving chickens that were challenged at 1 day old with strain ck/CH/LGX/11119. This finding is in agreement with the field cases, in which false layers were observed in the layer flock from which strain ck/CH/LGX/11119 was isolated.

The GI-28 type first emerged in 2005 in Guangdong province and has mainly circulated in south China in recent years (Fig. 5). Meanwhile, some strains of this type were also isolated in other regions of central China, implicating the wide distribution and spread of this IBV type and emphasizing the importance of continuous surveillance of IBV in chicken flocks in China. In addition, it is necessary to develop new live vaccines or evaluate the use of established vaccines in combination to control GI-28 type IBV strains in future because the GI-28 viruses related to cystic oviducts in layer flocks and might induce false layers.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2016.12.017>.

References

- Ababneh, M., Dalab, A.E., Alsaad, S., Al-Zghoul, M., 2012. Presence of infectious bronchitis virus strain CK/CH/LDL/971 in the middle east. *ISRN Vet. Sci.* 11.
- Ambali, A.G., Jones, R.C., 1990. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. *Avian Dis.* 34, 809–817.
- Ambali, A.G., 1992. Recent studies on the enterotropic strain of avian infectious bronchitis virus. *Vet. Res. Commun.* 16, 153–157.
- Awad, F., Chhabra, R., Forrester, A., Chantrey, J., Baylis, M., Lemiere, S., Hussein, H.A., Ganapathy, K., 2016. Experimental infection of IS/885/00-like infectious bronchitis virus in specific pathogen free and commercial broiler chicks. *Res. Vet. Sci.* 105, 15–22.
- Benyeda, Z., Mato, T., Suveges, T., Szabo, E., Kardi, V., Abonyi-Toth, Z., Rusvai, M., Palya, V., 2009. Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. *Avian Pathol.* 38, 449–456.
- Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. *Vet. Res.* 38, 281–297.
- Chen, L., Zhang, T., Han, Z., Liang, S., Xu, Y., Xu, Q., Chen, Y., Zhao, Y., Shao, Y., Li, H., Wang, K., Kong, X., Liu, S., 2015. Molecular and antigenic characteristics of Massachusetts genotype infectious bronchitis coronavirus in China. *Vet. Microbiol.* 181, 241–251.
- Coleman, C.M., Frieman, M.B., 2014. Coronaviruses: important emerging human pathogens. *J. Virol.* 88, 5209–5212.
- de Wit, J., Nieuwenhuisen-van Wilgen, J., Hoogkamer, A., van de Sande, H., Zuidam, G.J., Fabri, T.H.F., 2011. Induction of cystic oviducts and protection against early challenge with infectious bronchitis virus serotype D388 (genotype QX) by maternally derived antibodies and by early vaccination. *Avian Pathol.* 40, 463–471.
- Duff, R.H., McDonald, J.W., McMartin, D.A., Ross, J.G., 1971. Infection of day old chicks with infectious bronchitis (IB) virus and subsequent anatomical abnormalities. *Vet. Rec.* 85, 315.
- Feng, K., Xue, Y., Wang, F., Chen, F., Shu, D., Xie, Q., 2014. Analysis of S1 gene of avian infectious bronchitis virus isolated in southern China during 2011–2012. *Virus Genes.* 49, 292–303.
- Gao, M., Wang, Q., Zhao, W., Chen, Y., Zhang, T., Han, Z., Xu, Q., Kong, X., Liu, S., 2016. Serotype, antigenicity, and pathogenicity of a naturally recombinant TW I genotype infectious bronchitis coronavirus in China. *Vet. Microbiol.* 191, 1–8.
- Han, Z., Zhang, T., Xu, Q., Gao, M., Chen, Y., Wang, Q., Zhao, Y., Shao, Y., Li, H., Kong, X., Liu, S., 2016. 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. *Virus Res.* 213, 140–148.
- Hewson, K., Noormohammadi, A.H., Devlin, J.M., Mardani, K., Ignjatovic, J., 2009. Rapid detection and non-subjective characterisation of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model. *Arch. Virol.* 154, 649–660.
- Ignjatovic, J., Sapats, S., 2005. Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. *Arch. Virol.* 50, 1813–1831.
- Ignjatovic, J., Reece, R., Ashton, F., 2003. Susceptibility of three genetic lines of chicks to infection with a nephropathogenic T strain of avian infectious bronchitis virus. *J. Comp. Pathol.* 128, 92–98.
- Jackwood, M.W., Boynton, T.O., Hilt, D.A., McKinley, E.T., Kissinger, J.C., Paterson, A.H., Robertson, J., Lemke, C., McCall, A.W., Williams, S.M., Jackwood, J.W., Byrd, L.A., 2010. Emergence of a group 3 coronavirus through recombination. *Virology* 398, 98–108.
- Ji, J., Xie, J., Chen, F., Shu, D., Zuo, K., Xue, C., Qin, J., Li, H., Bi, Y., Ma, J., Xie, Q., 2011. Phylogenetic distribution and predominant genotype of the avian infectious bronchitis virus in China during 2008–2009. *Virol. J.* 22, 184.
- Liu, S., Kong, X., 2004. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Pathol.* 33, 321–327.
- Liu, X., Shao, Y., Ma, H., Sun, C., Zhang, X., Li, C., Han, Z., Yan, B., Kong, X., Liu, S., 2013. Comparative analysis of four Massachusetts type infectious bronchitis coronavirus genomes reveals a novel Massachusetts type strain and evidence of natural recombination in the genome. *Infect. Genet. Evol.* 14, 29–38.
- Liu, S.W., Zhang, Q.X., Chen, J.D., Han, Z.X., Liu, X., Feng, L., Shao, Y.H., Rong, J.G., Kong, X.G., Tong, G.Z., 2006. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. *Arch. Virol.* 151, 1133–1148.
- Luo, H., Qin, J., Chen, F., Xie, Q., Bi, Y., Cao, Y., Xue, C., 2012. Phylogenetic analysis of the S1 glycoprotein gene of infectious bronchitis viruses isolated in China during 2009–2010. *Virus Genes* 44, 19–23.
- Marandino, A., Pereda, A., Tomás, G., Hernández, M., Iraola, G., Craig, M.I., Hernández, D., Banda, A., Villegas, P., Panzera, Y., Pérez, R., 2015. Phylogenetic analysis of avian infectious bronchitis virus in South America. *J. Gen. Virol.* 96, 1340–1346.
- Masters, P.S., 2006. The molecular biology of coronaviruses. *Adv. Virus Res.* 66, 193–292.
- Mihindukulauriya, K.A., Wu, G., St Leger, J., Nordhausen, R.W., Wang, D., 2008. Identification of a novel coronavirus from a beluga whale by using a panviral microarray. *J. Virol.* 82, 5084–5088.
- Montgomery, R.D., Boyle, C.R., Maslin, W.R., Magee, D.L., 1997. Attempts to reproduce a runtling/stunting-type syndrome using infectious agents isolated from affected Mississippi broilers. *Avian Dis.* 41, 80–92.
- Moreno, A., Franco, G., Massi, P., Tosi, G., Blanco, A., Antilles, N., Biarnes, M., Majó, N., Nofrarías, M., Dolz, R., Lelli, D., Sozzi, E., Lavazza, A., Cecchinato, M., 2016. A novel variant of the infectious bronchitis virus resulting from recombination events in Italy and Spain. *Avian Pathol.* 22, 1–28.
- Mork, A.K., Hesse, M., Abd El Rahman, S., Rautenschlein, S., Herrler, G., Winter, C., 2014. Differences in the tissue tropism to chicken oviduct epithelial cells between avian coronavirus IBV strains QX and B1648 are not related to the sialic acid binding properties of their spike proteins. *Vet. Res.* 14, 45–67.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493–497.
- Reguera, J., Santiago, C., Mudgal, G., Ordoño, D., Enjuanes, L., Casasnovas, J.M., 2012. Structural bases of coronavirus attachment to host aminopeptidase N and its inhibition by neutralizing antibodies. *PLoS Pathog.* 8, e1002859.
- Satoh, R., Furukawa, T., Kotake, M., Takano, T., Motokawa, K., Gemma, T., Watanabe, R., Arai, S., Hohdatsu, T., 2011. Screening and identification of T helper 1 and linear immunodominant antibody-binding epitopes in the spike 2 domain and the nucleocapsid protein of feline infectious peritonitis virus. *Vaccine* 29, 1791–1800.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Valastro, V., Holmes, E.C., Britton, P., Fusaro, A., Jackwood, M.W., Cattoli, G., Monne, I., 2016. S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification. *Infect. Genet. Evol.* 39, 349–364.
- Wickramasinghe, I.N., van Beurden, S.J., Weerts, E.A., Verheije, M.H., 2014. The avian coronavirus spike protein. *Virus Res.* 194, 37–48.
- Woo, P.C., Lau, S.K., Lam, C.S., Tsang, A.K., Hui, S.W., Fan, R.Y., Martelli, P., Yuen, K.Y., 2014. Discovery of a novel bottlenose dolphin coronavirus reveals a distinct species of marine mammal coronavirus in Gammacoronavirus. *J. Virol.* 88, 1318–1331.
- Xu, C., Zhao, J., Hu, X., Zhang, G., 2007. Isolation and identification of four infectious bronchitis virus strains in China and analyses of their S1 glycoprotein gene. *Vet. Microbiol.* 122, 61–71.
- Ziebuhr, J., 2005. The coronavirus replicase. *Curr. Top. Microbiol. Immunol.* 287, 57–94.