



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



ELSEVIER

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Pathogenicity of a GI-22 genotype infectious bronchitis virus isolated in China and protection against it afforded by GI-19 vaccine

Ye Zhao¹, Deqiong Xie¹, Keran Zhang, Jinlong Cheng, Gang Xu, Guozhong Zhang*

Key Laboratory of Animal Epidemiology of the Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

ARTICLE INFO

Keywords:

Infectious bronchitis virus
GI-22 genotype
Pathogenicity
Protective efficiency

ABSTRACT

Avian infectious bronchitis (IB) is a globally circulating bird disease caused by infectious bronchitis virus (IBV). In China, the most prevalent IBV genotype is GI-19/QX-like because the protective efficiency of the classical IBV vaccine is low, and new GI-19 vaccines are under development. In 2018, a GI-22 genotype strain CK/CH/LGD/2018 (abbreviated 'LGD') was isolated in northern China, which caused 10%–30% mortality in H120-vaccinated chickens. A phylogenetic analysis showed that this new isolate displays novel features compared with other earlier-isolated reference strains. To monitor the epidemic trend of IBV in China, the pathogenicity of LGD was first evaluated in 1-day-old specific-pathogen-free chickens. LGD induced classical IBV damage in the trachea and kidney, whereas it also infected and damaged the bursa of Fabricius, an important immune organ of chickens. The efficacy of our earlier-developed GI-19 vaccine, strain SZ200, against LGD was also evaluated in this study. The GI-19 genotype vaccine provided sufficient protection against the new GI-22 genotype strain, and may be a promising candidate vaccine with which to control both wild GI-19 and GI-22 strains in the future.

1. Introduction

Avian infectious bronchitis (IB), caused by avian infectious bronchitis virus (IBV), is an acute and highly contagious disease that is extremely harmful to the poultry industry throughout the world (Cavanagh, 2007). The virus infects chickens of all ages, although chicks are most susceptible. It mainly infects the chicken respiratory and genitourinary systems, resulting in growth retardation and reproductive disorders (Cook et al., 2012; Zhong et al., 2016). Mixed infections with *Escherichia coli* ultimately lead to death, causing huge losses to the breeding industry.

IBV is a single-stranded positive-sense RNA virus. Because it has no polymerase proofreading mechanism, IBV is highly susceptible to mutation, and new serotypes and genotypes emerge constantly in the field (Capua et al., 1999; Zhao et al., 2016). Therefore, the virus exists as a wide range of antigenically and genetically distinct viral types, and the immune cross-protection offered by the different IBV serotypes is poor, making the prevention and control of this important pathogen both complex and challenging. Currently, over 50 different antigenic and genetic types of IBV are recognized, some of which have a substantial economic impact on the livestock industry and some of which are restricted to specific geographic areas (Capua et al., 1999; Wit et al., 2010). According to a new classification system based on the S1 gene

phylogeny of IBV, six genotypes comprising 32 distinct viral lineages and a number of interlineage recombinants have been identified (Valastro et al., 2016).

The largest number of IBV isolates in China are from the GI-19 lineage, also known as IBV strain QX/LX4-like, which was first detected in China in 1996, where it was predominantly associated with severe nephritis, 'false layer' syndrome, and potential proventriculitis (Wang et al., 1998; Xu et al., 2007; Zhao et al., 2016). Since then, QX-type strains have been identified in both China and Europe (Monne et al., 2008; Abro et al., 2012; Yan et al., 2017). Like GI-19, GI-22 is another frequently isolated genotype of IBV, which mainly circulated in the southern part of China before 2013. It mainly includes nephropathogenic field viruses collected from outbreaks in both broiler and layers flocks in 1997–2011 (Han et al., 2011). For unknown reasons, it has rarely been reported since then.

In this study, we identified a new IBV strain, CK/CH/LGD/2018 (abbreviated 'LGD'), isolated from northern China in 2018. A phylogenetic analysis showed it belongs to the GI-22 genotype, and its pathogenicity was investigated. In this study, we determined the protective efficiency of a representative attenuated live GI-19 vaccine, SZ200, against this new IBV strain. Our purpose was to provide a theoretical basis for the development of an immunization program for chicken farms and the better selection of vaccines that can contribute to the

* Corresponding author at: College of Veterinary Medicine, China Agricultural University, No. 2 Yuanmingyuan West Road, Haidian, Beijing, 100193, China.
E-mail address: zhanggz@cau.edu.cn (G. Zhang).

¹ These authors contributed equally to this study.

<https://doi.org/10.1016/j.virusres.2019.05.006>

Received 6 March 2019; Received in revised form 9 May 2019; Accepted 9 May 2019

Available online 10 May 2019

0168-1702/ © 2019 Elsevier B.V. All rights reserved.

prevention and treatment of IB.

2. Materials and methods

2.1. Viruses

IBV strain LGD originated from a H120-vaccinated broiler flock that had respiratory signs, severe renal disease, and a death rate of 10%–30%. The virus was purified and passaged by inoculating 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs via the allantoic sac route. The inoculated eggs were then incubated for 40 h at 37 °C, and the allantoic fluid was harvested for subsequent analysis. The GI-19 genotype vaccine strain, SZ200, was attenuated in our laboratory with 200 serial passages in SPF embryonated chicken eggs via the allantoic sac route. The safety and efficiency of SZ200 have been reported previously (Yan et al., 2018). Viral stocks of LGD and SZ200 were titrated by inoculating the allantoic sacs of 10-day-old SPF embryonated eggs with 10-fold serial dilutions of the viral stocks in phosphate-buffered saline, and the embryo 50% infectious doses (EID₅₀) were determined with the method of Reed and Muench (1938).

2.2. SPF chickens, chicken eggs, and ethics statement

SPF white leghorn chickens and SPF eggs were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd (Beijing, China). All the birds were kept in isolators at China Agricultural University (Beijing, China) throughout the experiments, and the animal-rearing facilities were approved by the Beijing Administration Committee of Laboratory Animals, under the auspices of the Beijing Association for Science and Technology (approval ID: SYXK [Jing] 2018–0038). All the animals used in this study were cared for in accordance with established guidelines, and the experimental protocols were performed with the approval of the Animal Welfare and Ethical Censor Committee of China Agricultural University.

2.3. Viral sequence analysis

The total RNA was extracted from 200 µl of allantoic fluid using the RNAPrep Pure Kit (For Tissue) (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions. Reverse transcription was conducted at 37 °C for 1 h, with 3 µg of total RNA, 1 µl of random hexamer primer (500 µg/ml; Promega, Madison, WI, USA), and 0.5 µl of M-MLV reverse transcriptase (200 U/µl; Promega). Twenty-eight primer pairs were used to PCR-amplify the complete genome of LGD (primer sequences available upon request). The PCRs were performed as described previously (Yan et al., 2017). All the amplified PCR products were analyzed with 1% agarose gel electrophoresis. Nucleotide sequencing was performed by TsingKe Biological Technology Co. (Beijing, China) using the Sanger sequencing method with the ABI 3730XL automatic sequencer (Applied Biosystems, USA).

The complete genomic sequence of the IBV LGD isolate was assembled with the DNASTAR software suite version 5.0 (DNASTAR, Madison, WI, USA). The assembled sequences of the complete genome and the S1 gene sequence were aligned with 49 complete IBV genomic reference sequences and 74 S1 gene sequences, respectively, downloaded from National Center for Biotechnology Information GenBank database, with MEGA6.0 software (www.megasoftware.net). Phylogenetic trees were constructed using the maximum likelihood method with the Kimura 2-parameter model in the same package, based either on the complete genome or the S1 gene. Bootstrap values were determined from 1000 replicates of the original data.

2.4. Pathogenicity experiment

Fifty 1-day-old SPF layer chickens were randomly assigned to one of four groups A, B, C, or D. Groups A and C (sampling groups) contained

15 birds each and groups B and D (clinical observation groups) contained 10 birds each. The LGD isolate was administered with eyedrop inoculation to the 1-day-old birds in groups A and B, at a dose of $1 \times 10^{6.0}$ EID₅₀ in a volume of 0.1 ml, and groups C and D were used as the negative controls.

2.5. Clinical observations in chickens

To determine the pathological characteristics of LGD, the birds in groups B and D were observed daily for 14 days postchallenge (dpc) for clinical signs, such as ruffled feathers, depression, respiratory signs, diarrhea, and mortality. The signs were scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe).

2.6. Sampling

At 3, 5, and 7 dpc, two birds from groups A and C were killed and necropsied. Any gross pathological changes were observed and tissue samples from the trachea, kidney, lung, and bursa of Fabricius were collected for virus detection with a reverse transcription–real-time quantitative PCR (RT–qPCR) assay or for histopathological examination. The tracheae of the necropsied chickens were also evaluated for tracheal ciliary activity. Ten nasopharyngeal swabs were collected from the birds in groups B and D at 3, 5, and 7 dpc. The swabs were placed in individual tubes containing 500 µl of sterile phosphate-buffered saline (pH 7.4) for viral detection with RT–qPCR.

2.7. Histopathology test

The tissues collected as described above were fixed in 10% neutral formalin for 24 h, embedded in paraffin, and stained with hematoxylin and eosin (HE) before they were observed with standard light microscopy. The lesions were scored according to their severity. The tracheal lesions were scored as follows: 2 for cilia loss, epithelial cell shedding, congestion, hemorrhage, and inflammatory cell infiltration; 1 for inflammatory cell infiltration and epithelial cell proliferation; 0 for normal histology. The lung lesions were scored as follows: 2 for diffuse alveolar and interstitial edema, inflammatory cell infiltration, hemorrhage, and necrosis; 1 for localized inflammatory cell infiltration and hemorrhage; 0 for normal histology. The kidney lesions were scored as follows: 2 for diffuse epithelial cell degeneration, necrosis and desquamation, renal tubular exudation, and inflammatory cell infiltration; 1 diffuse inflammatory cell infiltration; 0 for normal histology. The bursa of Fabricius lesions were scored as follows: 2 for cysts in the follicles with scattered lymphocytes, increased size of connective tissue, thickening and folded epithelium; 1 for follicles with moderate and generalized lymphoid depletion; 0 for normal histology. The mean lesion scores (MLSs) were calculated for each group.

2.8. Inhibition of ciliary activity

To evaluate tracheal ciliostasis, three sections each of the upper, middle, and lower parts of the trachea (nine rings per bird) were analyzed. The rings were placed in a Petri dish containing Eagle's minimal essential medium with 10% fetal bovine serum. They were then observed with an inverted light microscope at a magnification of 400×, to determine the degree of integrity and the preservation of ciliary movement on the tracheal epithelial cells. The average ciliostasis score was calculated for each group, as described previously (Zhao et al., 2015). A score of 0 was given if the cilia in the complete tracheal section showed movement; a score of 1 was given if 75%–100% of the cilia in the tracheal section showed movement; a score of 2 was given if 50%–75% of the cilia in the tracheal section showed movement; a score of 3 was given if 25%–50% of the cilia in the tracheal section showed movement; and a score of 4 was given if < 25% of the cilia in the tracheal section showed movement.

2.9. IBV detection with RT-qPCR

Total RNA was extracted from the tissues (trachea, kidney, lung, and bursa of Fabricius) and nasopharyngeal swabs as described above. The cDNAs were generated with the RT procedure described above. To quantify the viral load, SYBR Green I RT-qPCR was performed with the TB Green™ Premix Ex Taq (Takara Biomedical Technology, Japan) with primers specific to the 5' untranslated region (UTR), as described by Yan et al. (2016). All RT-qPCRs were performed in triplicate and repeated at least twice, and the IBV load was calculated with a standard curve.

2.10. Protective efficacy of GI-19 vaccine SZ200 against LGD

Seventy-five 14-day-old SPF chickens were divided into six groups of 15 or 10 birds. Groups A (15 birds) and B (10 birds) were used as the negative controls. Groups C (15 birds) and D (10 birds) were vaccinated intranasally with $10^{3.5}$ EID₅₀ SZ200 and challenged intranasally with LGD at a dose of $10^{6.0}$ EID₅₀/bird at 14 days postvaccination. Groups E (15 birds) and F (10 birds) were left unvaccinated and challenged with LGD at the same time. All the birds were housed separately in isolators under the same conditions, and food and water were provided ad libitum. After challenge, all the birds in groups B, D, and F were observed daily for 2 weeks for clinical signs attributable to IB infection. Two birds from groups A, C, and E were killed humanely at 3, 5, and 7 dpc to examine them for gross lesions and to evaluate their tracheal ciliary activity. The tracheae, kidneys, lungs, and bursas of Fabricius were collected from the birds in groups A, C, and E for viral detection with RT-qPCR. The methods used in these analyses were those described above.

2.11. Statistical analysis

Unless otherwise stated, all data are given as the means \pm standard deviations (SD) of experiments performed in at least triplicate. The survival curves were compared with a log rank test. The clinical scores, qPCR data on viral loads, and ciliary activity in the control groups and LGD challenged groups were compared with an unpaired Student's *t* test. The qPCR data on viral loads and the ciliary activities in the control group, SZ200-vaccinated group challenged with LGD, and unvaccinated group challenged with LGD were compared statistically with two-way ANOVA adjusted with a post hoc analysis, followed by Bonferroni's multiple-comparisons test. For all tests, the following notations are used to indicate significant differences between the groups: **p* < 0.05, ***p* < 0.01, ****p* < 0.001. All data were analyzed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Genetic characteristics of LGD genome

The complete genome of LGD is 27,591 nucleotides in length, including the 5'- and 3'-UTRs but excluding the poly(A) tail. The complete genomic sequence of LGD has the typical IBV gene order: 5'-UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3' UTR. Although LGD clustered with other QX-like strains on the phylogenetic tree based on complete IBV genomes (Fig. 1A), it shared low S1 gene similarity with the original QX strain (81.26%). However, its S1 gene shared relatively high genetic identity (93% according to BLAST) with that of the GI-22 genotype reference strain SAIBK (DQ288927), and shared even higher identity (99.82%) with that of strain gammaCoV/ck/China/11225/17-1 (MH397199), newly isolated in 2018. On the phylogenetic tree based on S1 sequences and constructed with the MEGA6.0 software (Fig. 1B), LGD clustered genetically with the GI-22 genotype, close to reference strains SAIBK and A2, and shared low homology with GI-19 genotype strain QX.

3.2. Pathogenicity of GI-22 genotype IBV LGD

The chickens challenged with LGD were observed for clinical manifestations for 1–14 days. The diseased chicks mainly showed signs of listlessness, huddling, ruffled feathers, and slight watery diarrhea. The signs were scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), and are shown in Fig. 2A. Of the 10 chicks tested, seven birds died between 4 and 12 dpc, and the mortality rate reached 70% (Fig. 2B). No obvious clinical signs or deaths attributable to IBV were observed in the control group. At necropsy, lesions were detected in the respiratory, urinary, and immune systems in the chickens inoculated with LGD, including punctate hemorrhage and catarrhal exudates in the throat and trachea at 3, 5, and 7 dpc. The lungs of the chickens showed congestion and edema at 3 and 5 dpc. The bursas showed heavy exudates of mucus and hemorrhage at 3, 5, and 7 dpc. The kidneys were swollen with urate deposits in the tubules and ureters at 3 dpc. No gross lesions were observed in any bird in the control group.

At 3, 5, and 7 dpc, two birds from groups A and C were euthanized, and their tracheae, kidneys, lungs, and bursas of Fabricius were resected for histopathological analysis. As shown in Fig. 3, at 3 dpc in group A, damaged tracheal epithelia and cilia shedding, with moderate lymphocyte infiltration, were observed. Severe bronchial hemorrhage was seen in the lungs, with numerous red blood cells in the lumen. Abundant lymphocyte infiltration was present in the tubulointerstitium region of the kidneys. No obvious lesions were found in the bursa of Fabricius. At 5 dpc, the mucosa of the trachea became thinner and the cilia had fallen off. Lymphocyte infiltration was present in the lobules of the lungs and more lymphocytes and inflammatory cells were observed in the lung atria, leading to the degeneration and narrowing of the entire alveolus pulmonis. In the kidney, the renal tubule epithelial cells were degenerated and necrotic, the renal tubule interstitia showed congestion, and the glomeruli showed occasional vasodilation and hyperemia. The number of lymphocytes in the follicles of the bursa of Fabricius was reduced. At 7 dpc, slight lymphocyte infiltration in the tracheal mucosa was observed, and the cilia of the villous layer had fallen off. The lung showed no obvious pathological changes. Lymphocyte infiltration was observed in the interstitia of the renal tubules. No obvious lesions were observed in the bursa of Fabricius. No lesions were observed in any tissue in control group C (Fig. 3A). The MLSs also showed that the lesions in the LGD-infected group were significantly more severe than those in the control group (Fig. 3B). The most severe lesions occurred at 5 dpc in all the organs, with MLSs of 1.67 ± 0.5 (trachea and lung), 1.78 ± 0.44 (kidney) and 1.44 ± 0.53 (bursa of Fabricius).

The inhibition of ciliary activity in the trachea was measured at 3, 5, and 7 dpc (Fig. 4A). The LGD-infected group showed a maximum average ciliostasis score of 4, whereas the average ciliostasis score in the control group was 0. The difference in ciliostasis between the LGD-infected group and the uninfected group was very highly significant (*p* < 0.001).

We determined the viral RNA titers in the tissues (trachea, kidney, lung, and bursa of Fabricius) and nasopharyngeal swabs with a SYBR Green I RT-qPCR assay. The viral loads in the different organs of the infected groups are shown in Fig. 4B. The viral DNA levels in the tracheae and lungs of the LGD-infected group peaked at 5 dpc and decreased at 7 dpc. The maximum amount of viral DNA was detected at 7 dpc in the kidneys of the LGD-infected group. The viral numbers in the bursa of Fabricius samples from the LGD-infected group peaked at 3 dpc. The viral DNA copy numbers were significantly greater in the trachea and kidneys than in the other organs at all time points. No virus was detected in the control group samples at similar time points.

We also tested the viral-DNA-positive rate in the nasopharyngeal swabs from both the infected and control groups. The proportions of positive samples in the group infected with the LGD strain (90%, 100%, and 100% at 3, 5, and 7 dpc, respectively) were significantly higher than those in the control group. No viral DNA was detected in the

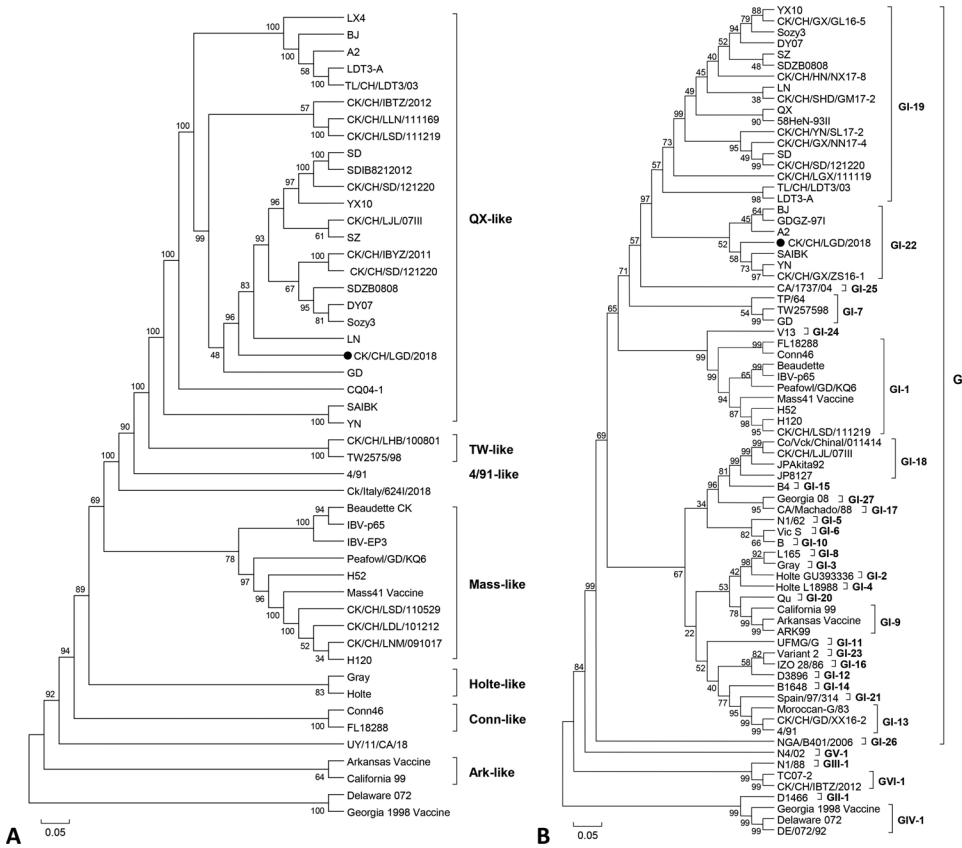


Fig. 1. Phylogenetic trees of LGD based on the complete genome (A) and the S1 gene (B). Forty-nine complete genome sequences and 74 S1 gene sequences from reference strains downloaded from NCBI were used separately in the analyses. The trees were constructed using the maximum likelihood method with the Kimura 2-parameter model and bootstrap tests with 1000 replicates in the MEGA6.0 program. Strain LGD used in this study is marked with a filled dot.

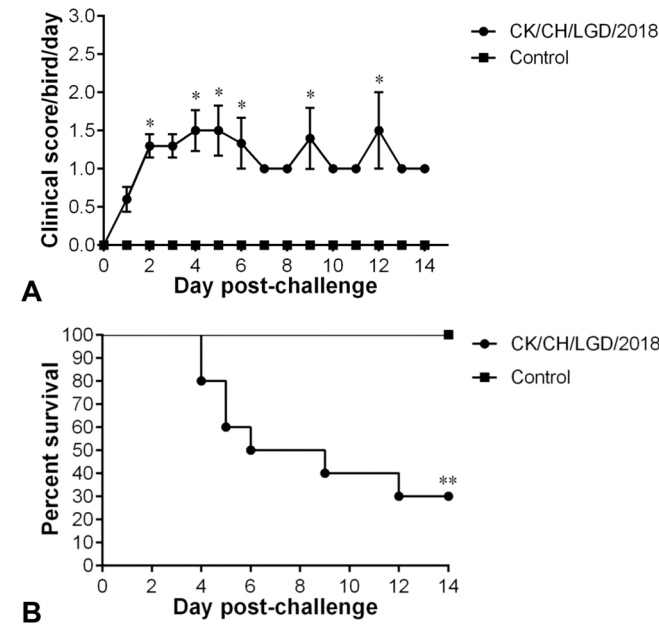


Fig. 2. Clinical scores (A) and percentage survival (B) in chickens experimentally infected with LGD and the control group (n = 10). Criteria for evaluation: 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Bars indicate means ± SD. Clinical scores were compared with an unpaired Student’s *t* test. Survival curves were compared with a log rank test. *Significant, $p < 0.05$; **highly significant, $p < 0.01$; and ***very highly significant, $p < 0.001$.

tissues or swabs from the mock-infected chickens.

3.3. Protective efficacy of GI-19 vaccine SZ200 against LGD

We investigated the protective efficiency of a representative attenuated live GI-19 vaccine, SZ200, against the new IBV strain LGD. SZ200 showed good protection against strain LGD. No clinical signs or deaths occurred in the SZ200-vaccinated group during the study period. Compared with the unvaccinated challenged group (60% morbidity, 10% mortality), the SZ200 vaccine reduced the morbidity and mortality of the chickens infected with LGD (Fig. 5A). The inhibition of the ciliary activity in the trachea was measured at 3, 5, and 7 dpc. The unvaccinated challenged group C showed a maximum average ciliostasis score of 4, whereas the average ciliostasis score in the SZ200-vaccinated group was < 2 (Fig. 5B). In terms of the clinical manifestations, no gross lesions were observed in the SZ200-vaccinated group. The difference in ciliostasis was extremely significant between the SZ200-vaccinated group and the unvaccinated group ($p < 0.01$). Significant reductions in the postchallenge viral load in the tissues of the SZ 200-vaccinated groups were detected at 3, 5, and 7 dpc compared with those in the unvaccinated group ($p < 0.05$) (Fig. 6).

4. Discussion

Infectious bronchitis is a highly infectious disease in poultry worldwide, and challenges the healthy development of the poultry industry (Cavanagh, 2005, 2007). Because of the unique primer transcription mechanism of the coronaviruses, the transcription and replication of IBV have high mismatch rates, generating many genotypic variants. In a previous study, we demonstrated that IBV is undergoing rapid evolution and display great variation, facilitated by multiple factors, including immunization pressures and environmental pressures (Zhao et al., 2016). At present, the IBV strains isolated throughout the world differ greatly in their pathogenicity, virulence, and tissue tropism

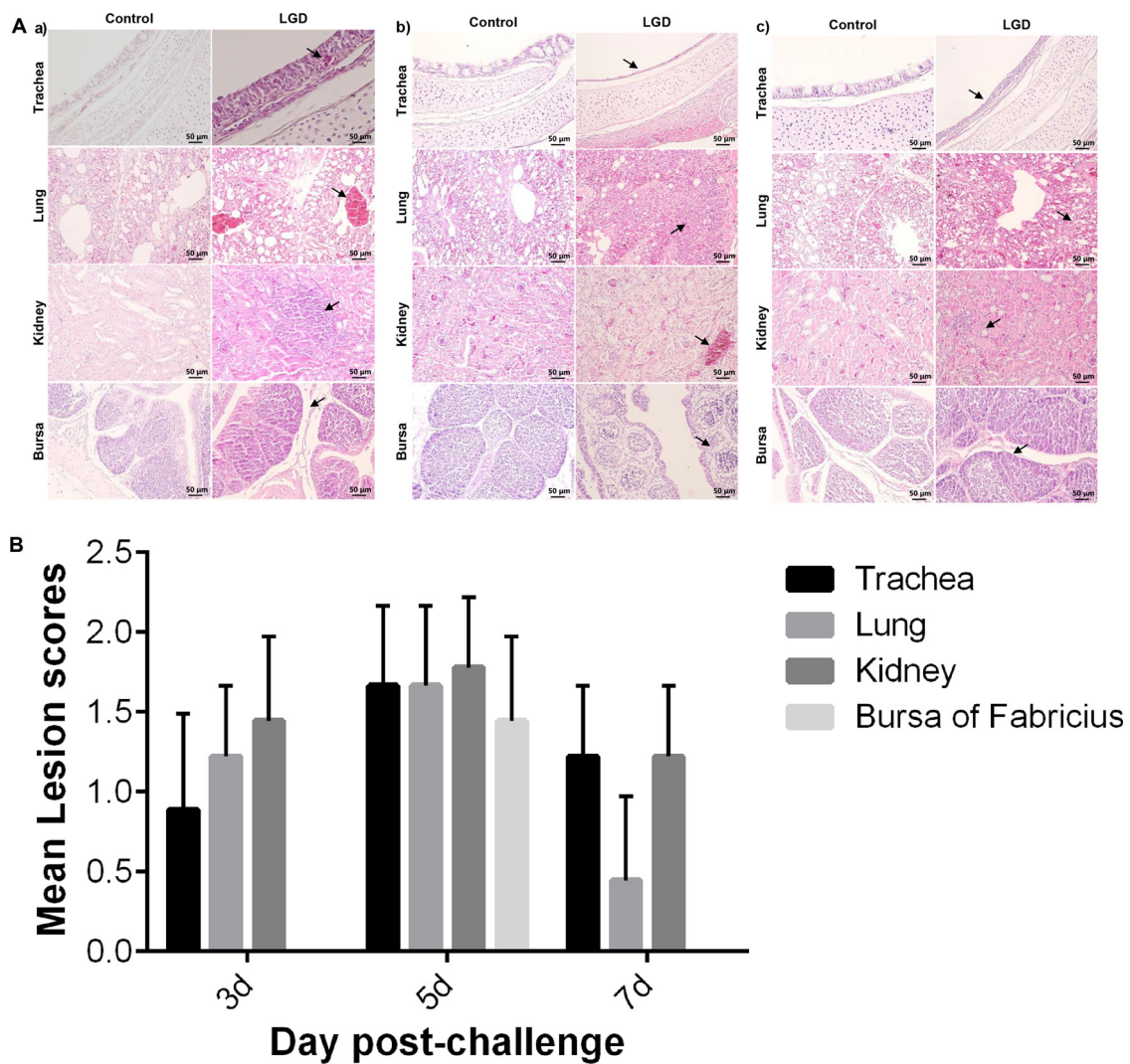


Fig. 3. Histopathological changes (A) and mean lesion scores (B) in the trachea, lung, kidney, and bursa of Fabricius at 3 (a), 5 (b), and 7 dpc (c) in chickens experimentally infected with LGD (group A) or the controls (group C). Black arrows indicate lesions detected in tissue. Scale bar = 50 μ m. Mean lesion scores were calculated according to the severity of the observed lesions in two chickens; nine blocks were chosen for each tissue sample.

(de Wit et al., 2011; Yan et al., 2017; Cheng et al., 2018), so it is necessary to monitor and explore the pathogenicity and immunological traits of IBV isolates to understand the current circulating trends in IBV.

According to the newly established phylogenetic analysis system based on the S1 gene, the recently isolated strain LGD belongs to the GI-22 genotype (Valastro et al., 2016). The Chinese IBV reference strain ck/CH/LSC/99I, isolated in 1999, was the earliest representative strain in the GI-22 lineage (Han et al., 2011). For unknown reasons, it has seldom been reported since 2013. In the present study, we found that although strain LGD belongs to the GI-22 genotype, it was clearly separated from the other GI-22 reference strains on a phylogenetic tree, and that it shares < 93.64% nucleotide identity with other GI-22 genotype reference strains, according to a BLAST comparison. However, it is noteworthy that LGD shares 99.82% identity with strain gammaCoV/ck/China/I1225/17-1 (MH397199), which was newly isolated and reported in 2018 (Xu et al., 2018). Moreover, an ⁶⁵ATTTA⁶⁹-C⁷¹ insertion was detected at positions 65–71 in the S1 gene of the LGD isolate and in gammaCoV/ck/China/I1225/17-1, relative to the S1 genes of the other GI-22 reference strains. This insertion could alter the immunogenicity and pathogenicity of strain LGD. The recent isolation of GI-22 strains with unique mutations indicates the possible circulation of these viruses in China, and the antigenic changes caused by this insertion in the viruses of the GI-22 lineage warrant further attention and investigation.

All IBV strains are considered to initially infect the respiratory tract, causing respiratory signs such as gasping, coughing, sneezing, tracheal rales, and nasal discharge (Cavanagh, 2005). Some IBV strains may involve nephrosis and nephritis and are referred to as ‘nephropathogenic strains’, and these may not produce significant respiratory lesions or clinical signs. In this study, we found that the newly isolated strain LGD mainly caused depression, huddling, ruffled feathers, increased water intake, and slight watery diarrhea during the observation period. The mortality rate was 60% in 1-day-old chickens. Gross lesions were detected in the respiratory, urinary, and digestive systems of chickens inoculated with LGD. As the target organs of IBV, the trachea and kidney were heavily damaged, as we expected, and this damage included punctate hemorrhage and catarrhal exudates in the throat and trachea. The kidneys were swollen with urate deposits in the tubules and ureters. HE-stained pathological sections of these tissues showed results consistent with the necropsy results. The viral loads in the trachea and kidney were higher than in the other organs. It is worth noting that the bursa of Fabricius displayed heavy exudates of mucus and hemorrhage at 3, 5, and 7 dpc and that the HE-stained sections showed reduced numbers of lymphocytes in the follicles of the bursa at 5 dpc. The viral loads in the bursa were determined with real-time PCR. There have been few reports of the damage caused to the bursa of Fabricius by IBV, although one paper reported that the bursa of

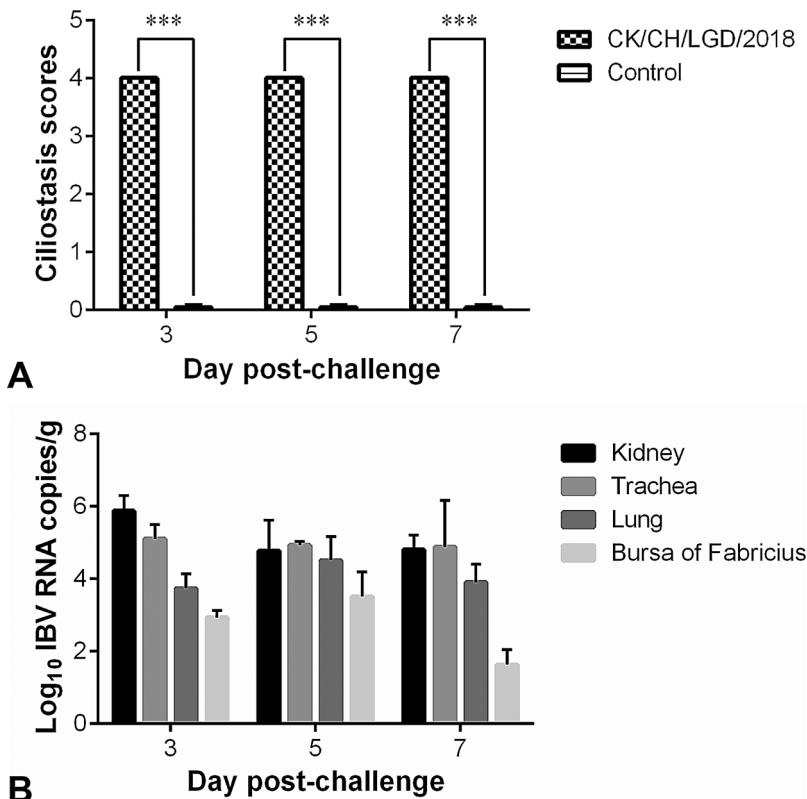


Fig. 4. Tracheal ciliostasis scores (A) and viral loads (B) in chickens experimentally challenged with LGD and controls. Bars indicate means \pm SD. Mean ciliostasis scores were calculated from the ciliostasis scores of two birds from each group euthanized at 3, 5, or 7 dpc (nine tracheal rings per individual bird). Score of 0: the cilia in the complete tracheal section showed movement; 1: 75%–100% of cilia in the tracheal section showed movement; 2: 50%–75% of cilia in the tracheal segment showed movement; 3: 25%–50% of cilia in the tracheal segment showed movement; 4: < 25% of cilia in the tracheal segment showed movement. The average ciliostasis score was calculated for each group. qPCR data for viral loads were calculated from two birds from each group euthanized at 3, 5, or 7 dpc, including three technical replicates per animal. Data were statistically compared with an unpaired Student's *t* test. *Significant, $p < 0.05$; **highly significant, $p < 0.01$; and ***very highly significant, $p < 0.001$.

Fabricius is not sensitive to IBV infection (with strains H120, M41, and SAIBK) (Fan et al., 2012). However, we have observed damage to the bursa of Fabricius caused by IBV infection in previous studies (Zhao et al., 2015; Yan et al., 2016), which is consistent with the results presented in this paper. Because it is an important immune organ of chickens, damage to the bursa of Fabricius can lead to strong immunosuppression in chickens (Gimeno and Schat, 2018), so the

immune function of IBV-infected chickens should be investigated in future studies.

The GI-22 genotype of IBV used in this study was isolated from chickens vaccinated twice with the H120 vaccine. The virus is probably able to evade the H120-induced immune response. The high genetic diversity among the endemic strains and the commercial vaccine strains of IBV results in increasing outbreaks of IB and a more complex control

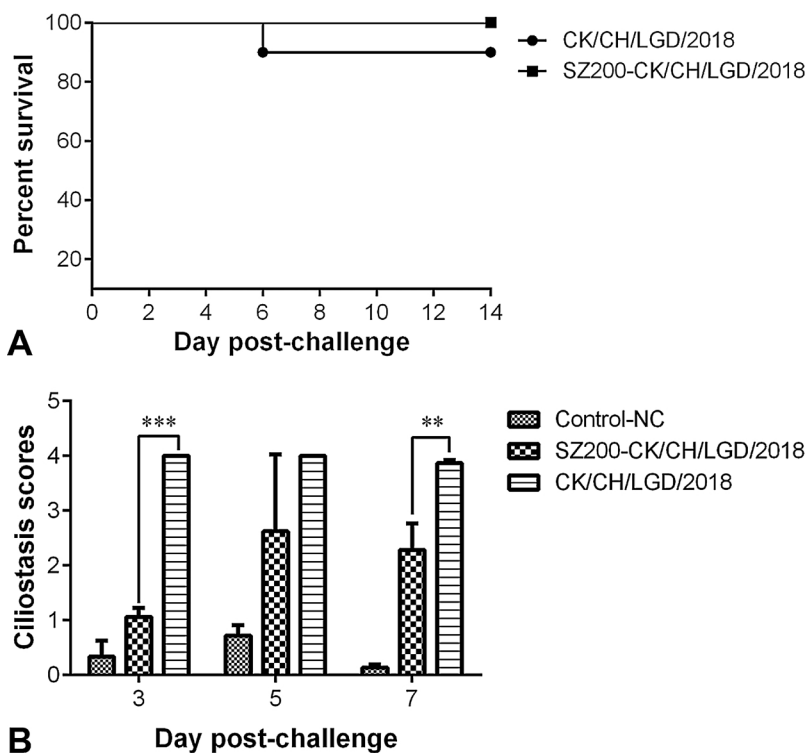


Fig. 5. Percentage survival ($n = 10$) (A) and tracheal ciliostasis scores ($n = 2$) (B) in vaccinated or unvaccinated chickens experimentally challenged with LGD and in the control group. Bars indicate means \pm SD. Survival curves were compared with a log rank test. Ciliostasis scores were statistically evaluated with two-way ANOVA adjusted with a post hoc analysis, followed by Bonferroni's multiple-comparisons test. *Significant, $p < 0.05$; **highly significant, $p < 0.01$; and ***very highly significant, $p < 0.001$.

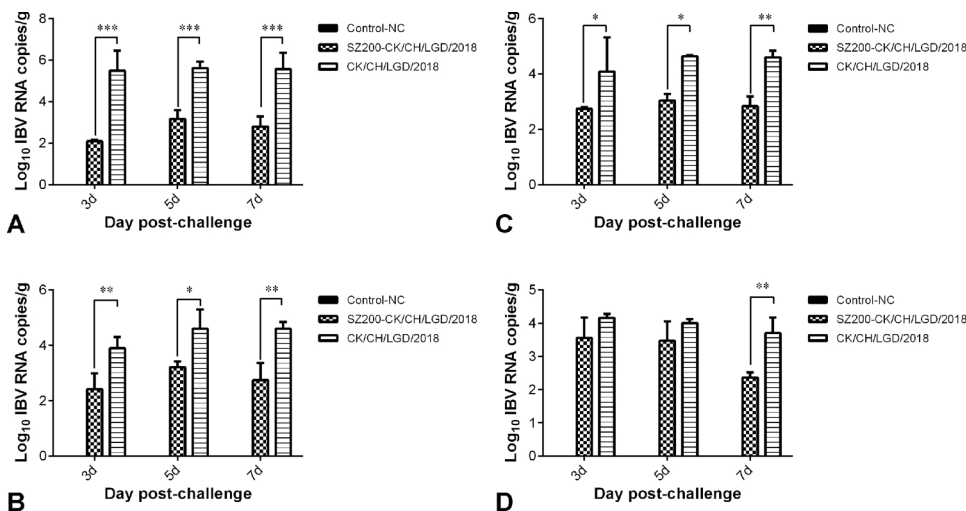


Fig. 6. Viral loads in the trachea (A), lung (B), kidney (C), and bursa of Fabricius (D) at 3, 5, and 7 dpc in chickens in the control group, SZ200-vaccinated chickens experimentally infected with LGD, and unvaccinated and challenged chickens (each $n = 2$). Viral loads were statistically evaluated with two-way ANOVA adjusted with a post hoc analysis, followed by Bonferroni's multiple-comparisons test. *Significant, $p < 0.05$; **highly significant, $p < 0.01$; and ***very highly significant, $p < 0.001$.

situation. Although Massachusetts IBV vaccines, such as H120, H52, and Ma5, have been widely used in the poultry industry, the immunized chickens are often not fully protected against wild IBV strains (Jiang et al., 2017). In a previous study, we showed that GI-19/QX-like IBV is the predominant strain in China (Zhao et al., 2016), so the preparation of a new candidate vaccine derived from GI-19/QX-like IBV may prove the best choice for IB control. We have developed a GI-19 genotype candidate vaccine, SZ200, which has displayed appropriate safety and immunogenicity in vivo experiments (Yan et al., 2018). Previous research has shown that although differences in S1 probably contribute to the failure to cross-protect some chicks, there may exist certain common epitopes between IBV isolates which have great importance in cross-immunity (Cavanagh et al., 1997). Information regarding the efficacy of vaccines against the IBV strains circulating in China will be valuable for the poultry industry when considering vaccine types. Therefore, we investigated the efficacy of the GI-19 genotype strain SZ200 to determine whether it provides protection against the virulent GI-22 strain LGD.

Our results indicate that strain SZ200 protected SPF birds against the morbidity and mortality induced by challenge with LGD. The SZ200-vaccinated group showed no clinical signs during the observation period after LGD challenge. Consistent with the clinical observations, no obvious gross lesions were detected in the different organs of the SZ200-vaccinated group. The quantification of ciliostasis (Cook et al., 1976; Cavanagh et al., 1997) or the determination of the viral loads in target organs are commonly used to assess chickens' immunity to challenge with IBV. The ciliostasis scores were clearly lower in the SZ200-vaccinated group than in the unvaccinated group after challenge with strain LGD, indicating that strain SZ200 effectively protected the respiratory tract. Significantly lower viral loads were also observed in the tissues of the vaccinated group after challenge, indicating that the SZ200 vaccine restricted LGD replication in different organs. These results indicate that GI-19 genotype vaccines can provide sufficient protection against the new GI-22 genotype strains identified so far, and may therefore offer promising candidate vaccines with which to control both wild GI-19 and GI-22 strains in the future.

In conclusion, we investigated the molecular characteristics and pathogenicity of the new GI-22 genotype strain LGD and identified new features in its S1 sequence and new tissue tropisms in this strain. We also investigated the protection conferred against LGD by GI-19 strain SZ200, with promising results. Our findings are of great importance for the prevention of IB.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by the National Key Research and Development Program of China (2017YFD0500700). We thank Janine Miller, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

References

- Abro, S.H., Renström, L.H., Ullman, K., Belák, S., Baule, C., 2012. Characterization and analysis of the full-length genome of a strain of the European QX-like genotype of infectious bronchitis virus. *Arch. Virol.* 157, 1211–1215.
- Capua, I., Minta, Z., Karpinska, E., Mawditt, K., Britton, P., Cavanagh, D., Gough, R.E., 1999. Co-circulation of four types of infectious bronchitis virus (793/B, 624/L, B1648 and Massachusetts). *Avian Pathol.* 28, 587–592.
- Cavanagh, D., 2005. Coronaviruses in poultry and other birds. *Avian Pathol.* 34, 439–448.
- Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. *Vet. Res.* 38, 281–297.
- Cavanagh, D., Elus, M.M., Cook, J.K., 1997. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. *Avian Pathol.* 26, 63–74.
- Cheng, J.L., Huo, C.Y., Zhao, J., Liu, T., Li, X., Yan, S.H., Wang, Z., Hu, Y.X., Zhang, G.Z., 2018. Pathogenicity differences between QX-like and mass-type infectious bronchitis viruses. *Vet. Microbiol.* 213, 129–135.
- Cook, J.K., Darbyshire, J.H., Peters, R.W., 1976. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. *Arch. Virol.* 50, 109–118.
- Cook, J.K.A., Jackwood, M., Jones, R.C., 2012. The long view: 40 years of infectious bronchitis research. *Avian Pathol.* 41, 239–250.
- de Wit, J.J., Cook, J.K.A., van der Heijden, H.M.J.F., 2011. Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathol.* 40, 223–235.
- Fan, W.Q., Wang, H.N., Zhang, Y., Guan, Z.B., Wang, T., Xu, C.W., Zhang, A.Y., Yang, X., 2012. Comparative dynamic distribution of avian infectious bronchitis virus M41, H120, and SAIBK strains by quantitative real-time RT-PCR in SPF chickens. *Biosci. Biotechnol. Biochem.* 76, 2255–2260.
- Gimeno, I.M., Schat, K.A., 2018. Virus-induced immunosuppression in chickens. *Avian Dis.* 62, 272–285.
- Han, Z., Sun, C., Yan, B., Zhang, X., Wang, Y., Li, C., Zhang, Q., Ma, Y., Shao, Y., Liu, Q., Kong, X., Liu, S., 2011. A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. *Infect. Genet. Evol.* 11, 190–200.
- Jiang, L., Zhao, W., Han, Z., Chen, Y., Zhao, Y., Sun, J., Li, H., Shao, Y., Liu, L., Liu, S., 2017. Genome characterization, antigenicity and pathogenicity of a novel infectious bronchitis virus type isolated from south China. *Infect. Genet. Evol.* 54, 437–446.
- Monne, I., Cattoli, G., Jones, R., Worthington, K., Wijmenga, W., 2008. QX genotypes of infectious bronchitis virus circulating in Europe. *Vet. Rec.* 163, 606–607.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* 27, 493–497.
- 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.
- Wang, Y.D., Wang, Y.L., Zhang, Z.C., Fan, G.C., Jiang, Y.H., Liu, X., Ding, J., Wang, S.S., 1998. Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. *Chinese Journal of Animal Quarantine* 1, 12–13.
- Wit, J.D., Cook, J., Heijden, H.V.D., 2010. Infectious bronchitis virus in Asia, Africa, Australia and Latin America: history, current situation and control measures. *Braz. J. Poultry Sci.* 12, 97–106.
- Xu, C.P., Zhao, J.X., Hu, X.D., Zhang, G.Z., 2007. Isolation and identification of four

- infectious bronchitis virus strains in China and analyses of their S1 glycoprotein gene. *Vet. Microbiol.* 122, 61–71.
- Xu, L., Han, Z., Jiang, L., Sun, J., Zhao, Y., Liu, S., 2018. Genetic diversity of avian infectious bronchitis virus in China in recent years. *Infect. Genet. Evol.* 66, 82–94.
- Yan, S.H., Chen, Y., Zhao, J., Xu, G., Zhao, Y., Zhang, G.Z., 2016. Pathogenicity of a TW-Like strain of infectious bronchitis virus and evaluation of the protection induced against it by a QX-Like strain. *Front. Microbiol.* 7, 1653.
- Yan, S.H., Liu, X.Y., Jing, Z., Gang, X., Ye, Z., Zhang, G.Z., 2017. Analysis of antigenicity and pathogenicity reveals major differences among QX-like infectious bronchitis viruses and other serotypes. *Vet. Microbiol.* 203, 167–173.
- Yan, S.H., Zhao, J., Xie, D.Q., Huang, X., Cheng, J.L., Guo, Y., Liu, C., Ma, Z., Yang, H.M., Zhang, G.Z., 2018. Attenuation, safety, and efficacy of a QX-like infectious bronchitis virus serotype vaccine. *Vaccine* 36, 1880–1886.
- Zhao, Y., Cheng, J.L., Liu, X.Y., Zhao, J., Hu, Y.X., Zhang, G.Z., 2015. Safety and efficacy of an attenuated Chinese QX-like infectious bronchitis virus strain as a candidate vaccine. *Vet. Microbiol.* 180, 49–58.
- Zhao, Y., Zhang, H., Zhao, J., Zhong, Q., Zhang, G.Z., 2016. Evolution of infectious bronchitis virus in China over the past two decades. *J. Gen. Virol.* 97, 1566.
- Zhong, Q., Hu, Y.X., Jin, J.H., Zhao, Y., Zhao, J., Zhang, G.Z., 2016. Pathogenicity of virulent infectious bronchitis virus isolate YN on hen ovary and oviduct. *Vet. Microbiol.* 193, 100–105.