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FULL PAPER
Avian Pathology

Identification of a novel avian coronavirus infectious bronchitis virus variant with three-nucleotide-deletion in nucleocapsid gene in China

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ABSTRACT. A novel avian infectious bronchitis virus (IBV) variant, designated as GX-NN160421, was isolated from vaccinated chicken in Guangxi, China, in 2016. Based on analysis of the S1 gene sequence, GX-NN160421 belonged to the New-type 1 (GVI-1) strain. More importantly, three consecutive nucleotides (AAC) deletions were found in the highly conserved structure gene N. The serotype of GX-NN160421 was different from those of the commonly used vaccine strains. The mortality of the GX-NN160421 strain was 3.33%, which contrasted with 50% mortality in the clinical case, but high levels of virus shedding lasted at least 21 days. In conclusion, the first novel IBV variant with three-nucleotide-deletion in the N gene was identified, and this unique variant is low virulent but with a long time of virus shedding, indicating the continuing evolution of IBV and emphasizing the importance of limiting exposure to novel IBV strains as well as extensive monitoring of new IBVs.

KEY WORDS: genotype, infectious bronchitis virus, N gene deletion, pathogenicity, serotype

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Avian infectious bronchitis (IB) is an important infectious disease that mainly affects the respiratory system of chickens, caused by infectious bronchitis virus (IBV). Some IBV strains can also affect the genitourinary or digestive system, causing the high mortality of sick chickens, decreased performance, reduced egg quantity and quality [24]. IBV belongs to coronavirus with a positive-sense, single-stranded, and its genome is prone to mutation and recombination. Due to a large number of IBV serotypes and the poor cross-protection between different serotypes, the currently used vaccines cannot provide complete protection, resulting in greater economic losses worldwide [23].

The structural proteins of IBV include the spike (S) glycoprotein protein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein [3]. The S protein contains S1 and S2 subunits, of which the S1 subunit determines the tissue affinity and virulence of IBV strains [3]. And the S1 subunit contains epitopes of inducing neutralization, hemagglutination inhibition, and serotype-specific antibodies [24]. The E protein is a hydrophobic virus pore protein that can form ion channels and promote the germination of virus particles [6]. Both M and E proteins are important for virus budding and the formation of virus-like particles [26]. The N protein mainly combines with the viral nucleic acid to form a nucleocapsid and participates in the synthesis, transcription, and translation of viral RNA, which is important in viral replication and assembly [4]. The N protein also carries some antigenic determinants and induces the body to produce antibodies and cellular immune responses [5].

The etiology of IB was firstly identified as IBV in 1936 [6]. So far, many IBV variants have been identified worldwide. Because of the extreme importance of the S1 protein, the S1 gene was often used as a target gene for molecular epidemiological analysis. Based on the S1 gene, IBV strains isolated in China were divided into at least eight genotypes of Mass-type (GI-1), Taiwan-type (GI-7), 4/91-type (GI-13), LX4-type (GI- 19), CK/CH /LSC/99I-type (GI-22), LDT3-A-type (GI-28), New-type 1 (GVI-1), New-type 2 (GVII-1) [25]. New-type 1 (GVI-1) and New-type 2 (GVII-1) strains emerged in China in recent years, and the New-type 2 (GVII-1) showed change in pathogenicity [25].

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It is known that the IBV variants arose via point mutations, insertions and deletions of nucleotides, and recombinations. Compared to the S1 gene, the N gene is relatively conservative. However, recent studies have shown that there are also large mutations in the N gene [2, 26, 27, 29, 31]. Among these descriptions, point mutations of N gene were the most frequently reported [2, 26, 29, 31], and there is only one report on the insertion of N gene [27]. There were no previous reports on the deletion of N gene. In this study, a New-type IBV strain with three nucleotides (one amino acid) deletion in the N gene was isolated and identified from vaccinated chicken with obvious acute respiratory symptoms in Guangxi, China, in 2016, and its genotype, whole genome, serotype, and pathogenicity were analyzed. This study reported for the first time that three nucleotides (one amino acid) deletion was found in the conservative N gene of IBV.

MATERIALS AND METHODS

Eggs and chickens

The specific-pathogen-free (SPF) white leghorn eggs were purchased from Beijing Merial Vital Laboratory Animal Experiment Technology Co., Ltd., Beijing, China. Four day-old SPF white leghorn chickens were purchased from Zhejiang Lihua Agricultural Science and Technology Co., Ltd., Yuyao, China. The animal experiment was approved by the Animal Care & Welfare Committee of Guangxi University (License no. GXU2018-026), and animal ethics guidelines were strictly implemented.

Clinical information, isolation and identification of virus

The virus was isolated from the 30-day-old local breed of chickens with obvious symptoms of acute respiratory disease in Guangxi, China, in April 2016. The diseased chickens, which were vaccinated with live vaccine strain H120 at 6 and 16 days old, showed symptoms of depression, coughing, gasping, craning neck, and closing eyes. The morbidity of the diseased birds was 90% and the mortality was 50%. Gross examination of sick birds revealed serous exudate in the trachea, and bursa atrophy was also found in some sick birds.

The trachea and kidney tissues of the sick birds were collected aseptically. The collected tissues were grinded and inoculated into 9-day-old SPF chicken embryo via the allantoic cavity route and blind passage three times were performed. The allantoic fluid was used for RT-PCR detection based on the 3′ untranslated region (3′UTR) of IBV as our previous description [10]. At the same time, the liver tissue was collected for bacteria isolation and culture.

Purification of virus

In order to get monocle IBV, the limiting dilution passage was conducted in chicken embryos according to the previous description [34]. Briefly, 0.2 ml allantoic fluid diluted to 10^2–10^9 times was inoculated into each 9-day-old SPF chicken embryo (5 embryos for each dilution). The collected allantoic fluid was detected IBV by RT-PCR as our previous description [34]. The allantoic fluid, which was obtained from the embryos inoculated with the highest dilution ratio and positive for IBV, was used for the next dilution passage. After three limiting dilution passages, the allantoic fluid positive for IBV was selected for the complete genome sequencing, serotype identification, and pathogenicity test.

High-throughput sequencing of the complete genome

The complete genome sequence of the isolate was obtained by high-throughput sequencing conducted by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China). The genome sequence of the isolate has been deposited in the GenBank database under the accession number MW222189.

Sequence alignment and phylogenetic tree analysis based on the S1 and N genes

The sequences of S1 and N genes were compared with those of 82 reference strains retrieved from the GenBank database (Supplementary Table 1). Alignment and similarity analyzes of S1 and N gene sequences were conducted used EditSeq and MegAlign programs in the Lasergene package (DNAStar, Madison, WI, USA). The phylogenetic trees of S1 and N genes were constructed by the neighbor-joining method using MEGA version X software, with 1,000 bootstrap replicates.

In addition, the deduced amino acid sequence of the S1 gene of the isolate was compared with those of the common vaccine strains H120, M41, 4/91, LDT3-A, and QXL87 in China. The amino acid sequences of three hypervariable regions (HVR I, HVR II, HVR III) and the minimal receptor-binding domain (RBD) were also compared as the previous description [32]. The EditSeq program was used for the S1 protein cleavage site analysis of the isolate.

Genome sequence comparison and phylogenetic tree analysis based on the complete genome sequences

Alignment analysis and phylogenetic tree construction of the complete genome sequence were performed for the isolate and 85 reference strains, including the above mentioned 82 reference strains as well as one strain of turkey and two strains of duck (Supplementary Table 1).

Recombination analysis

Multiple sequence alignment of the complete genome sequences of the GX-NN160421 and the reference strains was performed with Multiple Alignment with Fast Fourier Transformation (MAFFT). Seven algorithms of recombination detection program (RDP4) software (RDP, GENECONV, Bootscan, MaxChi, Chimera, SiScan, and 3Seq) were used for gene recombination analysis according to the previous study [26]. The window size was adjusted to 40 bp, and the highest acceptable *P* value was 0.05.

Serotype identification

The tracheal organ culture (TOC) virus neutralization (VN) test was used to identify the serotype of the isolate. The monovalent antisera of GX-YL5 (serotype I), vaccine strain H120 (serotype II), GX-C (serotype III), GX-YL1 (serotype IV), GX-NN7 (serotype V), GX-YL9 (serotype VI), GX-NN12 (serotype VII) prepared and stored by the Institute of Poultry and Poultry Diseases, Guangxi University [22] were used for the VN tests. The preparation of TOCs of chicken embryos, determination of median tracheal organ cultures infective dose (TOC-ID_{50}), and the VN tests were carried out as our previous description [22]. The highest serum dilution that could neutralize virus, which caused ciliostasis and beaten cilia was the neutralization titer. The viruses that share the same neutralization titer or titer only with one dilution difference are taken as the same serotype.

Pathogenicity test

Sixty 7-day-old SPF chickens were randomly divided into 2 groups, namely the GX-NN160421 group and the control group. Birds in the GX-NN160421 group were infected with 10^6 EID_{50} of the GX-NN160421 strain by the nasal-ocular route, and the birds in the control group were given an equal volume of sterile PBS in the same way. Clinical signs were observed and recorded daily. Clinical signs were observed and recorded daily. Five birds were euthanized and necropsied at 3, 5, 7, 11, 14, and 21 days post-infection (dpi), respectively. The trachea, lung, and kidney tissues were collected and fixed in 10% neutral formalin for histopathology analysis according to the previous description [15]. The trachea, kidney tissues, and cloacal swabs were collected for the detection of viral loads by real-time PCR as previously described [15].

Animal and human rights statement

The authors confirm that the ethical policies of the journal, as noted on the journals author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

RESULTS

Virus isolation and identification

The SPF chicken embryos were found to die within 96 hr after three blind passages and showed dwarfing, stunting, and curling appearance. The allantoic fluids of each passage were performed hemagglutination assay (HA) and they showed no HA activity for chicken red cells. The RT-PCR amplification of the 3′UTR of IBV showed a positive result. The bacteria isolated from liver were consistent with the characteristics of *Staphylococcus aureus* and *E. coli*.

Complete genome and organization of GX-NN160421 strain

The high-throughput sequencing results showed that the GX-NN160421 strain had a genome-wide nucleotide sequence length of 27,751 bp, and was consistent with the classic IBV genome structure 5′-UTR-Pol-S-3a-3b-E-M-5a-5b-N-UTR-3′ (Table 1). The length of the S gene was 3,513 bp and the S glycoprotein was cleaved into S1 and S2 subunits, which were 1,635 bp and 1,878 bp, respectively. The length of the N gene was 1,227 bp. The gene annotation of the complete genome sequence of the GX-NN160421 strain was shown in the Table 1. Three consecutive nucleotides (AAC) deletion at 26,458–26,460 bp was found in the N gene of the complete genome (Supplementary Material), and this nucleotides deletion caused one amino acid deletion when compared with the commonly used vaccines Fig. 1.

Table 1. Gene annotation of complete genome sequence of GX-NN160421 strain

Gene		Genome position	Size (nucleotide)	Size (amino acid)
5′-UTR		1–525	525	—
1ab polyprotein	NSP2	526–2,547	2,022	673
	NSP3	2,548–7,317	4,770	1,589
	NSP4	7,318–8,865	1,548	515
	NSP5	8,866–9,786	921	306
	NSP6	9,787–10,668	882	293
	NSP7	10,669–10,917	249	82
	NSP8	10,918–11,547	630	209
	NSP9	11,548–11,810	333	110
	NSP10	11,811–12,315	435	144
	NSP11	12,316–12,384	69	22
	NSP12	12,316–15,134	2,819	930
	NSP13	15,135–16,934	1,800	599
	NSP14	16,935–18,497	1,563	520
	NSP15	18,498–19,511	1,014	337
	NSP16	19,611–20,420	810	269
Spike glycoprotein	S1	20,371–22,005	1,635	545
	S2	22,006–23,883	1,878	625
3a protein		23,883–24,056	174	57
3b protein		24,056–24,244	189	62
Envelope protein		24,225–24,536	312	103
Membrane protein		24,526–25,206	681	226
5a protein		25,570–25,767	198	65
5b protein		25,764–26,012	249	82
Nucleocapsid protein		25,955–27,181	1,227	408
3′-UTR		27,182–27,751	570	—

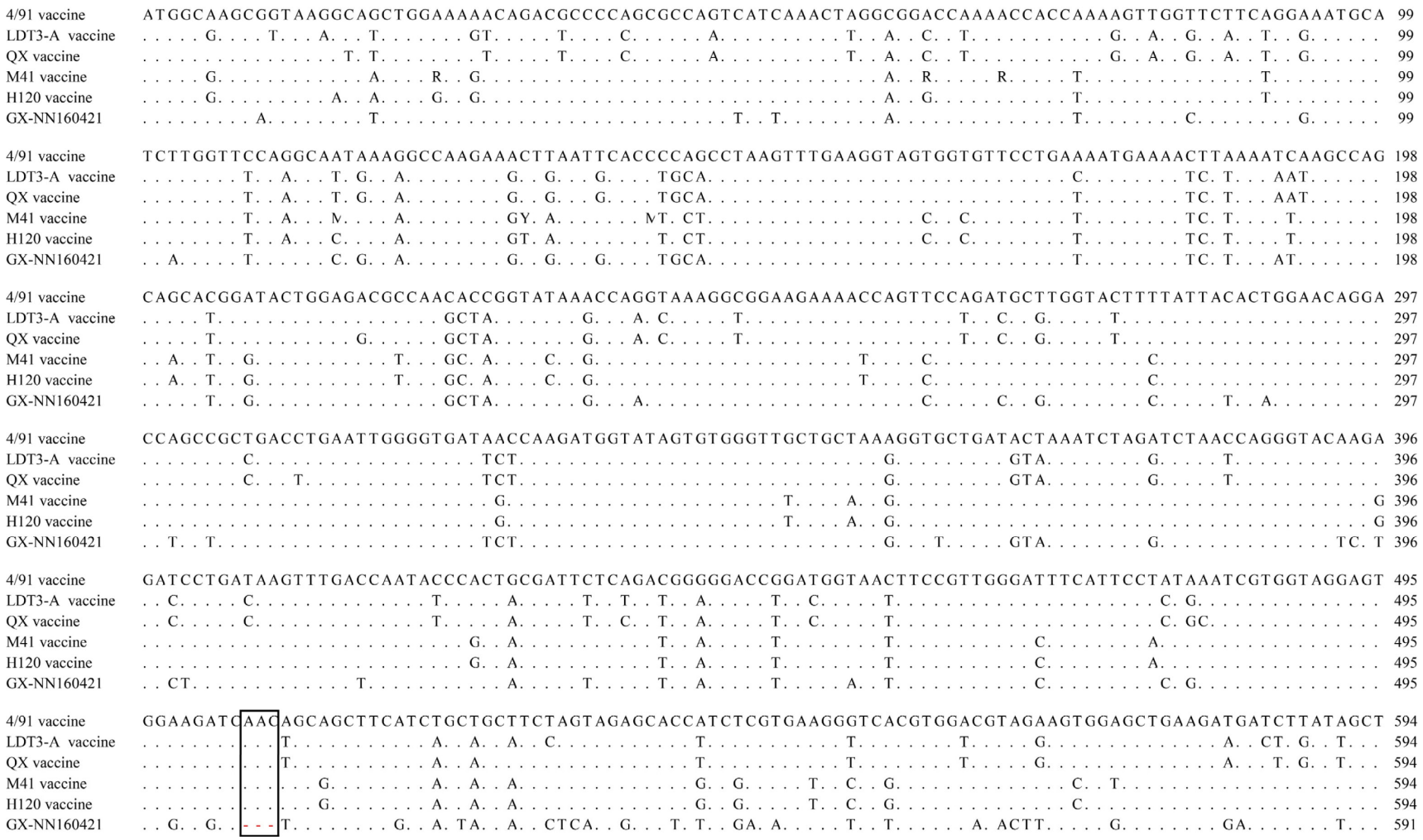


Fig. 1. The nucleotide sequence comparison of N gene of GX-NN160421 isolate and N gene of IBV vaccine 4/91, H120, LDT3-A, M41, QX strain. The N gene deletion site of GX-NN160421 isolate is boxed by black lines.

Sequence alignment and phylogenetic tree analysis of S1 and N genes

The nucleotide and deduced amino acid sequence similarities of the S1 and N genes between the GX-NN160421 and the 82 IBV reference strains were 38.4–97.6%, and 62.4–99.1%, respectively. The similarities of nucleotide and deduced amino sequences of S1 gene between the GX-NN160421 strain and the vaccine strains H120, H52, Ma5, 28/86, M41, W93, 4/91, LDT3-A and QXL87 were 64.8% (aa: 58.6%), 64.9% (aa: 58.2%), 64.9% (aa: 58.4%), 64.9% (aa: 58.4%), 65.0% (aa: 58.8%), 64.7% (aa: 58.2%), 65.4% (aa: 58.7%), 66.0% (aa: 59.7%) and 66.3% (aa: 60.9%), respectively.

The phylogenetic tree results showed that the GX-NN160421 isolate had the highest similarity (99.0%) with the Chinese isolates I0636-16 and GX-NN09032, belonging to the New-type 1 (GVI-1) (Fig. 2). The same reference strain was also applied to the N gene phylogenetic tree analysis. Based on the N gene, the GX-NN160421 isolate belonged to CK/CH/LSC/99I genotype (Fig. 3).

In addition, the amino acid comparison results of the S1 gene of the GX-NN160421 and five commonly used vaccine strains 4/91, H120, LDT3-A, M41, QXL87 in Guangxi showed that there were four aa (QKEP) insertions in HVR III, and multiple mutations of GX-NN160421 were distributed uniformly over the N-terminal region of the S1 subunit (Fig. 4). The S glycoprotein cleavage site of the GX-NN160421 strain was HRRKR.

Sequence alignment and phylogenetic tree analysis of complete genome

The phylogenetic tree at the complete genome level was shown in Fig. 5. The GX-NN160421 isolate had the closest genetic relationship with the I1209/16 (97.7% genome nucleotide identity) and GX-NN09032 (96.4% genome nucleotide identity) strains, and they belonged to the same group.

No recombination event in GX-NN160421 strain

Recombination events of the GX-NN160421 isolate were examined used the RDP software in this study. The results of seven algorithms of RDP4 software analysis showed that there was no evidence of recombination within the genes of S1, N, and at the genome-wide level.

The serotype of GX-NN160421 is different from those of the vaccine strains

The serotype identification results showed that the GX-NN160421 isolate shared the highest neutralization titers with the GX-C strain (serotype III) (Table 2). Therefore, the GX-NN160421 isolate belongs to serotype III and was different from the serotypes of the commonly used vaccine strains H120 and 4/91.

GX-NN160421 is a low virulent strain with virus shedding at least 21 days

Clinical signs of all birds were observed after the challenge. The majorities of birds in the GX-NN160421 group showed clinical symptoms such as depression, loose feathers, coughing, tracheal rales, and increased drinking water. Mucus and hemorrhage were observed in the trachea, but no obvious urate deposition in the kidney. A bird in the challenge group died at 2 dpi. The morbidity rate was 76.67% (23/30), and the mortality rate was 3.33% (1/30). No clinical signs and gross lesions were observed in the birds of the control group.

Histopathological examination revealed that the tracheal mucosa was edematous and loss of cilia, minor infiltration of lymphocytes were observed at 3dpi; sloughing of epithelial cells and massive infiltration of lymphocytes were observed at 5 dpi to 11 dpi; regeneration of the epithelium and cilia recovery was observed at 14 dpi, but there was still minor infiltration of lymphocytes in the tracheal mucosa (Fig. 6). Sloughing of epithelial cells and massive infiltration of lymphocytes were observed in the bronchial mucosa of the lung. Local pulmonary congestion, pulmonary chamber compression atrophy was observed at 11dpi-14dpi (Fig. 7). No microscopic changes were observed in the kidney of birds from the GX-NN160421 group. No microscopic changes were observed in the trachea, lung, and kidney of birds from the control group.

The virus loads in the trachea, kidney, and cloacal swabs were analyzed during 3–21 dpi. As shown in Fig. 8 the trachea virus loads gradually increased from 3 dpi to 5 dpi, peaked at 5 dpi, and gradually decreased from 7 dpi to 21 dpi. The levels of viral RNA in the trachea were significantly higher than those in the kidney from 3 dpi to 14 dpi ($P<0.05$). The viruses were detected in the cloacal swabs from 3 dpi and peaked at 11 dpi, but kept in high levels at all the time points and was still very high with a viral copy number of around 1,268.198 copies/μl at 21 dpi. No virus was detected from the birds in the control group.

DISCUSSION

Due to the discontinuous replication of IBV RNA, the genome is prone to mutation and recombination, causing the changes of genotype, serotype, and pathogenicity. Although the commercial IBV vaccines had been used worldwide, the IB continues to be a challenging problem, and IBV variants have been continually isolated and identified [1, 7, 11, 13, 18, 23, 25, 28,]. Among the identified variants, some strains occurred mutation and recombination in the S1 gene; some showed change in antigenicity; and some in pathogenicity. In this study, we reported a novel genotype IBV strain with three consecutive nucleotide deletions in the N

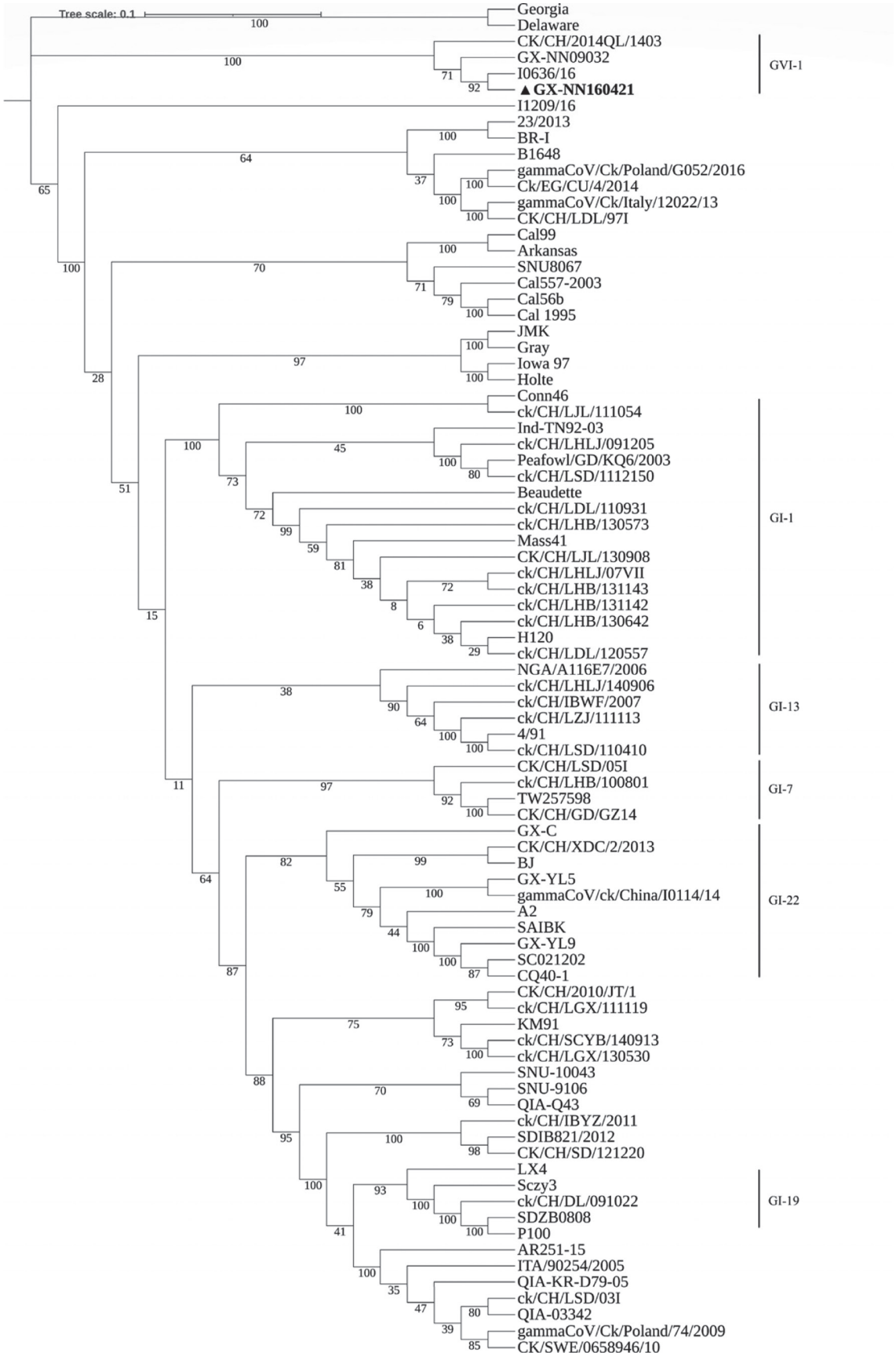


Fig. 2. Phylogenetic tree base on the S1 gene sequences of GX-NN160421. The GX-NN160421 isolate is marked with a black triangle. The branch number represents the percentage of times that the branch appeared in the tree.

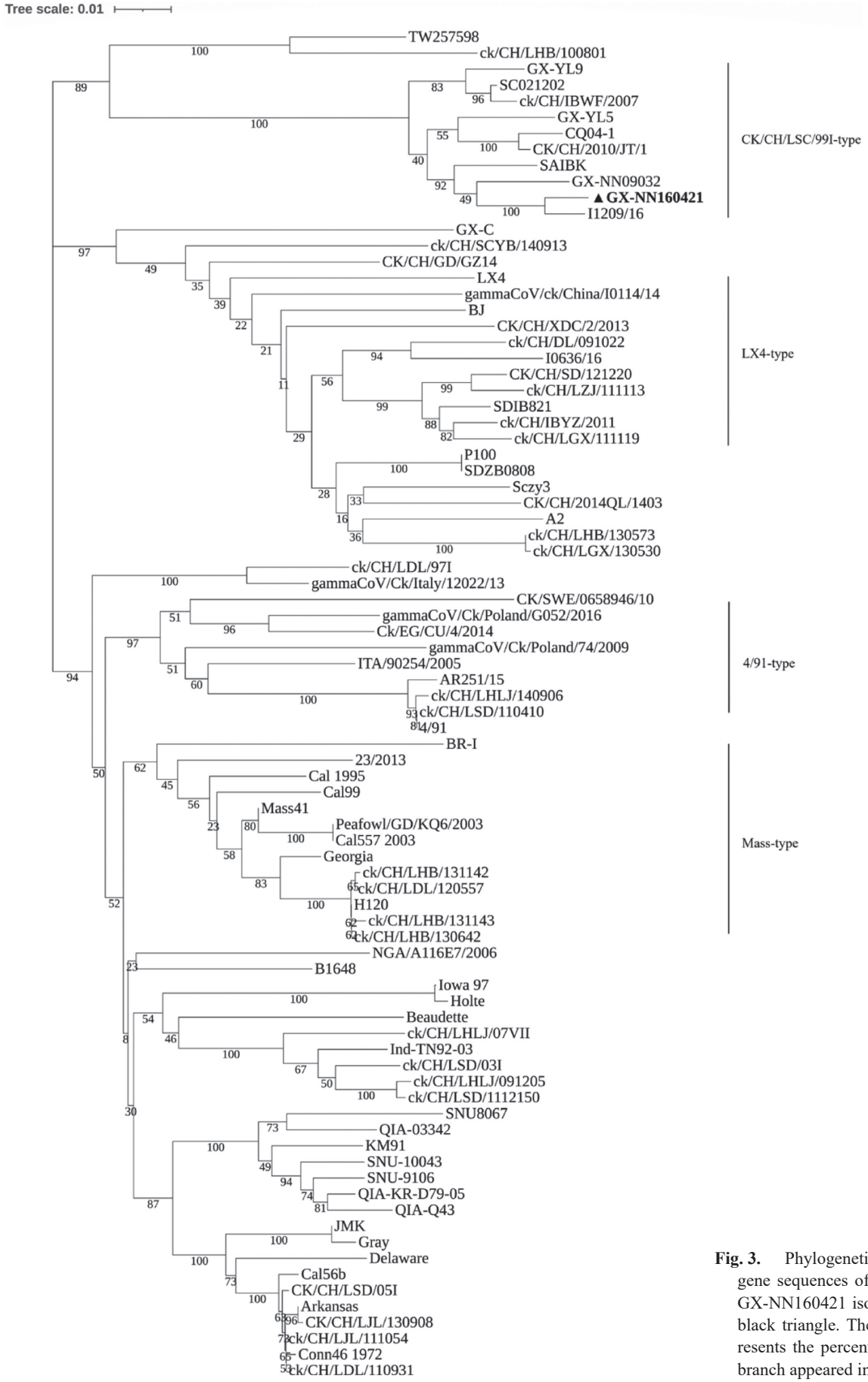


Fig. 3. Phylogenetic tree base on the N gene sequences of GX-NN160421. The GX-NN160421 isolate is marked with a black triangle. The branch number represents the percentage of times that the branch appeared in the tree.

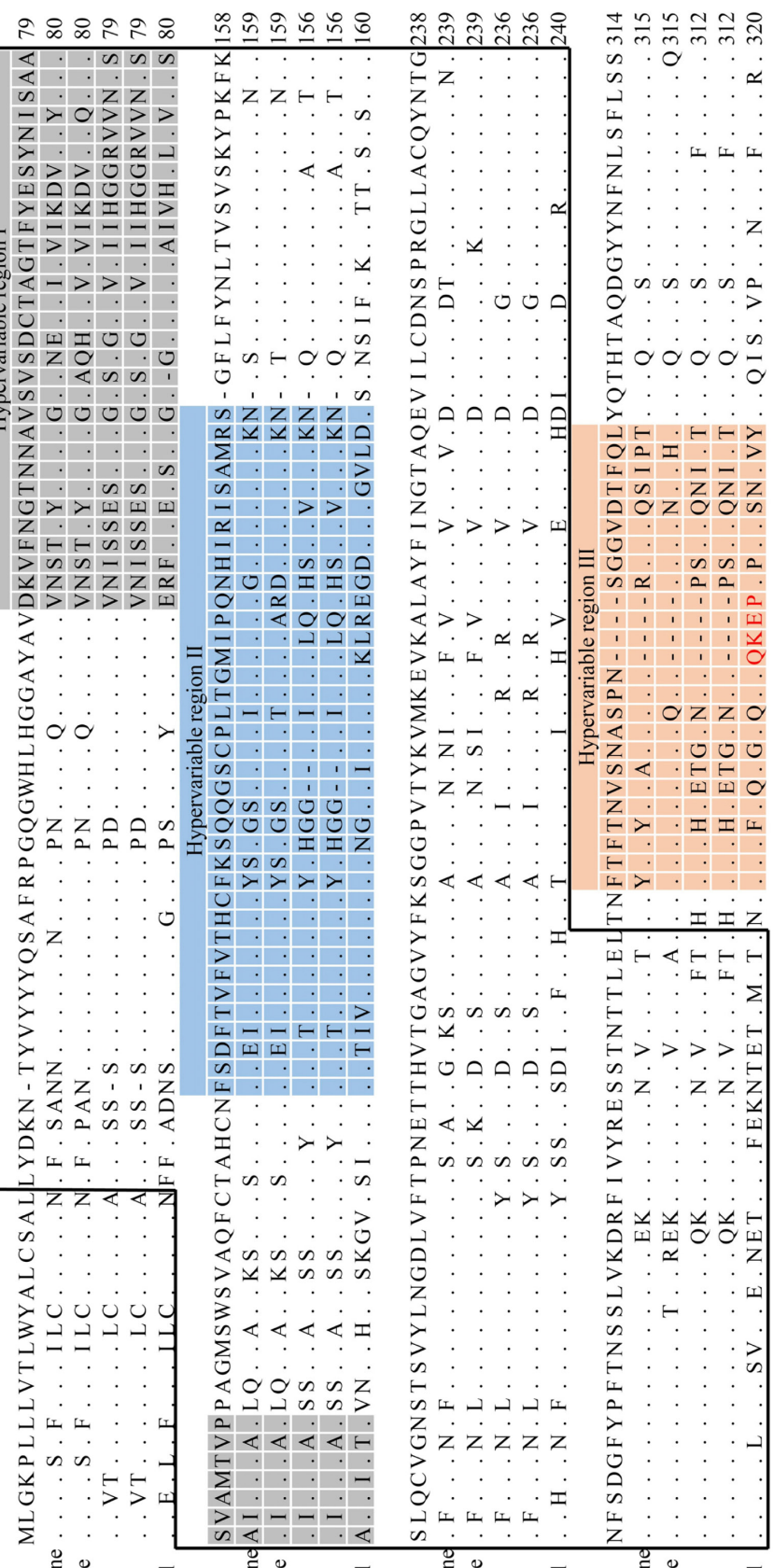
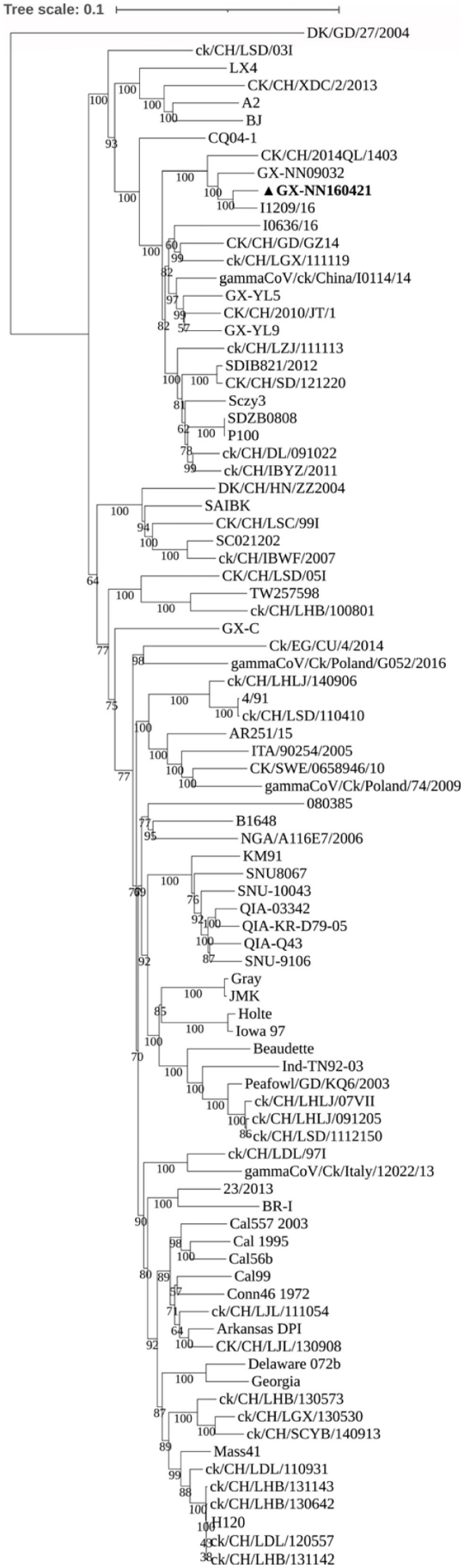


Fig. 4. Comparision of amino acid sequences of S1 protein from GX-NN160421 isolate and IBV vaccine strains 4/91, H120, LDT3-A, M41, and QXL87. The amino acid sequences encoding the three hypervariable regions (HVR I, HVR II, HVR III) are shown in background colors. The minimal receptor-binding domain corresponding to the most N-terminal residues 20-271 of the spike protein of IBV 4/91 is boxed.



gene, and the strain is low virulent but sheds high-level viruses for a long time (at least 21 days).

Among the four structural protein genes of IBV, the S1 gene is most prone to mutation, and it is closely related to serotype, pathogenicity, and producing neutralizing antibodies [19]. Therefore, the genetic analysis of IBV is mainly based on the S1 gene. In the current study, the complete S1 gene of GX-NN160421 was used for IBV genotyping and the result showed that the S1 genotype of strain GX-NN160421 was New-type 1 (GVI-1), and the strain GX-NN160421 shared nucleotide and amino acid similarities with vaccine strain H120 was only 64.8% and 58.6%, respectively. In addition, there were four consecutive amino acids (QKEP) insertions in HVR III, of GX-NN160421. The four consecutive amino acids were also observed in other strains such as GX-NN09032, and they belonged to the same genotype [14]. The amino acid mutation in the HVR of the S1 gene maybe lead to the occurrence of new serotype and immunity escape [25]. The serotype of the GX-NN160421 strain was further confirmed to be different from the commonly used vaccine strains H120 and 4/91. Whether the change of serotype is related to the four aa (QKEP) insertions in HVR III needs further study.

In addition to S1 gene, there were reports on mutations in the N gene in recent years [2, 26, 27, 29, 31]. Among these studies, most the observed mutations in the N gene were point mutations. There is only one previous study on the insertion of N gene [27], which reported five amino acid sequences SLNRG were inserted into the two Korean IBV isolates. However, the pathogenicity of two Korean IBV isolates from the above mentioned report on the insertion of N gene was not observed [27]. No previous report on the deletion of N gene is available. This may imply that the 3 consecutive nucleotides deletion in N gene found in GX-NN160412 was occurred during egg passages in the laboratory. However, in our study this possibility is low because the N gene was amplified from the allantoic fluid only after three blind passages. The previous study showed that three blind passages didn't cause nucleotides deletion [33]. The mutations in the N gene might result in changes in N protein epitope and function, which might alter certain biological characteristics of the virus [27]. In our study, an IBV strain GX-NN160421 with three consecutive nucleotides (one amino acid) deletion at 26,458–26,460 bp in the N gene was firstly isolated and identified. Whether the three consecutive nucleotides deletion in the N gene of the GX-NN160421 strain is related to the changes of virus epitope and biological characteristics remains unclear. The pathogenicity of GX-NN09032 was not high [8], but GX-NN09032 had the highest similarity with GX-NN160421 in terms of nucleotide sequences of N genes, and GX-NN09032 did not have 3 consecutive nucleotides deletion in the N gene. To some extent, it can be said that 3 consecutive nucleotides deletion in the N gene did not play an essential role in the low pathogenicity of GX-NN160421. But whether this is the case remains to be further studied. In addition, we noticed that GX-NN09032 belonged to serotype 5 [8], which is different from the serotype 3 of GX-NN160421. The similarities of N, S1 and the whole genome sequence between GX-NN160421 and GX-NN09032 are 96.5%%, 98.3%, and 96.4%, respectively. There were so high similarities between GX-NN160421 and GX-NN09032, but the two strains shared different serotypes, suggesting that a few amino acids or even point mutations may affect serotypes or other biological activities. Therefore, the 3 consecutive nucleotides deletion in the N gene of GX-NN160421 may have a relationship with the epitope and other biological

Fig. 5. Phylogenetic tree base on the complete genome sequences of GX-NN160421. The GX-NN160421 isolate is marked with a black triangle. The branch number represents the percentage of times that the branch appeared in the tree.

Table 2. Data on neutralizing antibody titers against the 7 serotype strains

Virus	Neutralization titers of the virus against the antisera						
	Serotype $1_{(GX\text{-}YL5)}$	Serotype $2_{(H120)}$	Serotype $3_{(GX\text{-}C)}$	Serotype $4_{(GX\text{-}YL1)}$	Serotype $5_{(4/91)}$	Serotype $6_{(GX\text{-}YL9)}$	Serotype $7_{(GX\text{-}NN12)}$
GX-NN160421	1:16	1:16	1:256	1:16	1:16	1:32	1:32

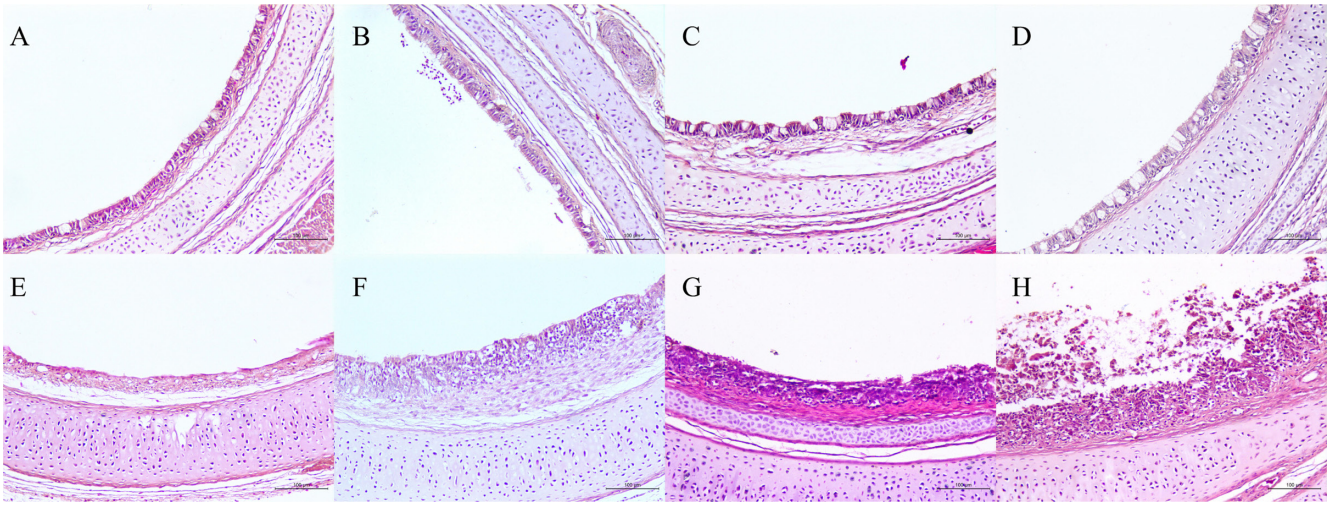


Fig. 6. Histopathologic lesions in trachea tissues from chickens infected with GX-NN160421 strain. Panels **A**, **B**, **C**, and **D** correspond to the trachea tissues of the control group at 3 dpi, 5 dpi, 7 dpi and 11 dpi, respectively. **E)** The trachea tissue with edematous mucosa, rounding epithelial cells, and loss of cilia at 3 dpi. **F)** The trachea tissue with disordered mucosa, denatured and necrotic epithelial cells, and massive infiltration of lymphocytes at 5 dpi. **G)** The trachea tissue with disordered mucosa, denatured and necrotic epithelial cells, lymphocytic infiltration, congestion and edema of submucosal vascular at 7 dpi. **H)** The trachea tissue with extensive sloughing, degeneration, and necrosis of epithelial cells at 11 dpi. Scale bar=100 µm.

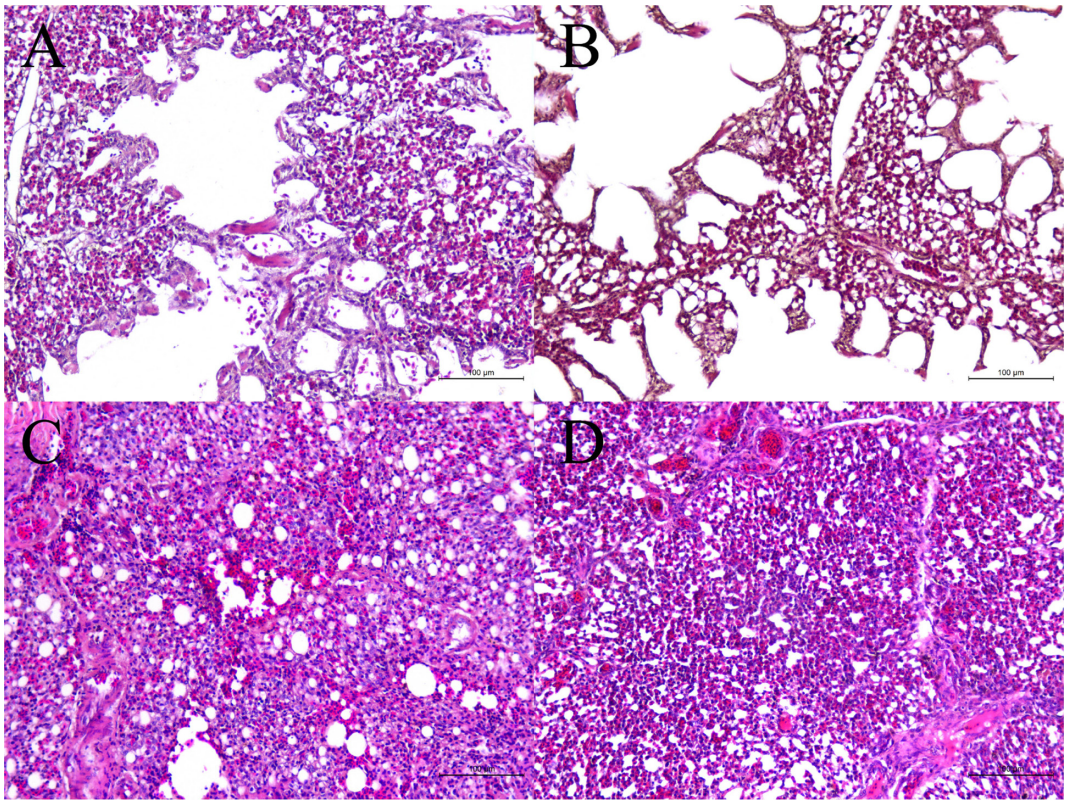


Fig. 7. Histopathologic lesions in lung tissues from chickens infected with GX-NN160421 strain. Panels **A** and **B** correspond to the lung tissues of the control group at 11 dpi and 14 dpi, respectively. **C)** The lung tissue with massive infiltration of lymphocytes at 11 dpi. **D)** The lung with congestion and lymphocytic infiltration at 14 dpi. Scale bar=100 µm.

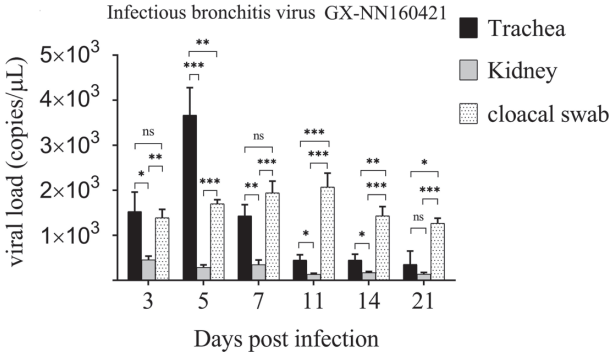


Fig. 8. Infectious bronchitis virus viral loads in trachea, kidney and cloacal swab of chickens infected with GX-NN160421. Statistical significance was considered using two-way ANOVA as follows: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, $P>0.05$.

characteristics. An in-depth and comprehensive study of this strain is necessary further.

Recently, the pheasant coronaviruses (PhCoV) I0710/17, genetically similarity to IBV, were isolated from commercial pheasants were found to be non-pathogenic to SPF chickens but highly pathogenic to commercial pheasants, with obvious clinical signs and 30% mortality [12]. Interestingly, the N gene of the PhCoV I0710/17 is 1,227bp, indicating that there is three nucleotides deletion in the N gene. However, the deletion in the N gene in the PhCoV has not attracted more attention and the exact location of the deletion in the N gene of PhCoV was also not indicated in the report.

So far, there is no information on the pathogenicity and virus shedding of IBV with the deletion in N gene. In the animal pathogenicity test, chickens infected with the GX-NN160421 showed mild respiratory symptoms with 76.67% morbidity and 3.33% (1/30) mortality, which contrasts with the 90% morbidity and 50% mortality in the field case. A similar case has been previously reported [17]. The possible reason might be that the IBV strain GX-NN160421 was not the only causative pathogen for the clinical outbreak. In our study, the diseased birds suffered IBDV infection at 20 days old and were conducted castration at 24 days old [20], which causing immunosuppression and heavy stress to chickens and further reduced the resistance of the birds, making them prone to infection with IBV and causing damage to the respiratory mucosa. In addition, the chickens were raised at a rather high density and the serious environmental pollution provided conditions for the secondary infection of *Staphylococcus aureus* and *E. coli*. (Via the respiratory tract infection or castration wound infection) and horizontal transmission. As a result, high morbidity and high mortality occurred in the field case. Hence, in addition to the appropriate immunization schedule, the control of immunosuppressive disease is very important for the control of IBV. In addition, good biosafety and management practices also play important roles in the prevention and control of IB.

In addition, the levels of viral RNA in the trachea were significantly higher than those in the kidney, agreeing with gross lesions and histopathological examination in the two organs. The challenge birds shed high-level viruses during the experiment even at 21dpi, although the clinical signs disappeared at that time point. The cloacal swabs were only observed for 21 days in our study, but the level of viral RNA was still very high at 21dpi, so it is estimated that the time for virus shedding would be longer than 21 days. Although high titers of the virus were confirmed in cloacal swabs, no clinical diarrhea was observed. IBV was excreted in large quantities from intestines to feces, which may be an important source for the continuous production of new variants [21]. Therefore, the recovered birds from the disease would become the dangerous infection source in the farm, especially in the yard or the free-range farm. Although the GX-NN160421 strain was low virulent causing the respiratory signs, it should be paid attention to due to the long time of virus shedding. In addition to S1 gene mutation, whether the long time of virus shedding is related to the deletion of the N gene remains unclear and needs further study.

Recombination is one of the contributing factors for IBV variation. In the present study, the genotypes of the S1 and N gene were different. Unexpectedly, there was no evidence of recombination at the S1gene, N gene, and genome-wide level, although the S1 and N genes shared different genotypes. A similar phenomenon was seen in other previous descriptions [35]. Therefore, the difference in genotypes between genes should not be regarded as evidence of recombination events but should be measured by the results of recombination analysis. A similar viewpoint was stressed in other previous studies [9, 16, 30]. In addition, there were reports that the mutations of the S1 gene and the N gene were almost independent of each other [27], which seems to explain why the genotypes of the S1 gene and the N gene of GX-NN160421 were not the same but no recombination event occurred.

In conclusion, the first novel genotype IBV strain with three-nucleotide-deletion in the N gene was identified in the present study, and the IBV strain is low virulent but sheds virus for a long time, indicating that IBV continues to evolve through mutation in conserved N gene besides the S1 genes and emphasizing the importance of limiting exposure to novel IBV strains as well as regular and extensive monitoring of new IBVs.

CONFLICT OF INTEREST. The authors declare that they have no conflict interest.

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