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WHOLE GENOME SEQUENCING ANALYSIS OF AVIAN INFECTIOUS BRONCHITIS VIRUS ISOLATED IN HUNG YEN PROVINCE IN 2021

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SUMMARY

Infectious bronchitis is an acute disease in chickens caused by the Infectious Bronchitis Virus (IBV). The disease causes serious economic losses in many countries in the world that have chicken farming industries. Even though vaccines have been used, the disease is still very complicated in many provinces and cities in Vietnam. The reason is that the virus strains causing the disease in the field have many changes compared to the vaccine virus strains. Research showed that there are numerous strains and genotypes of IBV in Vietnam. However, only the S1 gene has been studied, and no research has been done on the full genome. In this study, the IBV strain causing disease in 2021 in Hung Yen Province (North Vietnam) was sequenced and analyzed for molecular characteristics. The analysis results showed that strain IBHYM belongs to genotype QX-like (GI-19) and has a close relationship with Chinese IBV strains. The IBHYM strain has higher nucleotide and amino acid similarities with strains of genotype QX-like (87.5% to 95% for nucleotide and 80.2% to 91.3% for amino acid) and the lowest similarity to strains of genotype TC07-like (70.4% to 70.5% for nucleotide and 53.8% to 56.2% for amino acid). The field pathogenic virus strains also have low nucleotide and amino acid similarity with the Massachusetts vaccine strain currently in use (86% for nucleotide and 77.1% for amino acid). Research results show the importance of molecular epidemiological surveillance and the selection of appropriate vaccine strains in Vietnam. This is the first report of sequencing the whole IBV genome in Vietnam.

Keywords: *Hung Yen province, IBV, genome, genotype*

INTRODUCTION

Infectious bronchitis (IB) is a contagious respiratory disease in chickens caused by the Infectious Bronchitis Virus (IBV). Chickens of all ages are susceptible to IBV, and when infected, they show clinical signs such as tracheal rales, panting, coughing, runny

nose, sneezing, and facial swelling (Cook, 2007). Due to its high incidence, wide distribution, reduced egg production and quality, and increased mortality when strains causing kidney disease or secondary infections occur, IBV causes great economic losses in the industry (Jackwood, de Wit, 2013). IBV infection is estimated to be the third most damaging cause of all livestock diseases, after avian influenza and echinococcosis (World Bank, 2011).

IBV belongs to the genus Gammacorona virus in the family *Coronaviridae*, order *Nidovirales* (Cavanagh, Britton, 2008; Jack Wood, Wit, 2013). The IBV genome is a positive single-stranded RNA that is about 27 kb in length. It codes for four structural proteins, including spike glycoprotein (S), envelope protein (E), membrane glycoprotein (M), and nucleoprotein (N). IBV's spike glycoprotein is about 1170 amino acids in length and goes through a process called post-translational cleavage to make S1 and S2 subunits with about the same mass. The S1 subunit, which has the neutralizing viral epitope, is more stable than the S2 subunit, which has the most variable region and is strongly linked to viral virulence (Cavanagh, Davis, 1986; Ismail *et al*., 2001). Nucleotide sequences are heterogeneous in the S1 gene and prominent in three hypervariable regions (HVRs) (amino acids 38–67, 91–141, and 274–387) (Cavanagh *et al*., 1988; Moore *et al*., 1997). Studies around the world focus mainly on the complete or partial S1 gene sequence to determine the genetic type of the virus. However, comprehensive genomic analysis is the best method to determine the true origin and evolutionary history of IBV (Reddy *et al*., 2015).

Many serotypes and variations of IBV have been found worldwide since it was originally characterized in 1936. To date, at least 32 serotypes based on the S1 gene have been found globally; the most prevalent are QX, 4/91, LDT3, HN08, TW, TC07-2, and Mass. They are caused by nucleotide point mutations, insertions, and/or deletions in the genome, particularly in the S1 subunit of the S protein (Cavanagh, 2007). Because of the lack of cross-protection between IBV serotypes, vaccination of hens is problematic, resulting in large economic losses in the poultry sector (Cavanagh, 2007; Lin, Chen, 2017).

In Vietnam, IB disease has received research attention in recent years. The reports have shown that IBV in Vietnam includes many different strains and genotypes, exactly like in some nearby nations like China and Malaysia. Using molecular biology methods, studies have detected the circulation of IBV strains of the Massachusetts serotype (lineage H120) and 793/B (lineage 4/91) on industrial chicken farms in Lam Dong province (Vo *et al*., 2012); serotype 793/B, QX-like in Can Tho (Tran *et al*., 2017); strains Q1-like, QX-like, and TC07-02-like in Hanoi and some northern provinces (Nguyen *et al*., 2017). The data from a portion of the S1 gene was used in all of the above experiments to identify the virus. There has been no published research in Vietnam that has decoded the whole genome. As a result, we conducted this study to decode and analyze the full genome of the IBV strain in Hung Yen, Vietnam as a scientific basis for contributing to the development and usage of IBV vaccines in Vietnam.

MATERIALS AND METHODS

Sample collection

The samples (throat fluid and kidney

tissue) were taken from affected chickens at an outbreak in Hung Yen province in 2021. Samples were homogenized in phosphate-buffered saline (pH 7.2) and frozen and thawed three times. The homogenate was then centrifuged at 5000 × g for 5 minutes at 4ºC, and the supernatant fluid was collected for further use.

Total RNA extraction

Total viral RNA was extracted from the supernatant fluid of the processed sample using the RNeasy Micro Kit (QIAGEN, Germany) following the manufacturer's instruction.

Detection of IBV using one-step RT-PCR

Total RNA was tested for the presence of IBV by RT-PCR reaction using the detected primers IBF: TTTTGGTGAYGAYAARATGA and IBR: TGCATTGTTCCTCTCYTC. This is a primer pair to amplify a part of N gene. The RT-PCR reactions were performed using the OneTaq® One-Step RT-PCR Kit (NEB, New England Biolabs).

Reverse transcription

cDNA synthesis was conducted using the Maxima First Strand cDNA Synthesis Kit (Thermo, Mỹ) as follows: 1 pg–5 µg of total RNA, 4 µL of 5X reaction mix, 2 µL of Maxima Enzyme Mix, water nuclease-free up to 20 µL. The cDNA synthesis reaction mixture was incubated at 25°C for 10 minutes, followed by 15 min at 50°C and then for 5 min at 85°C to inactivate the enzyme. The cDNA samples were stored at -20°C for further use.

Primers and PCR amplification

The primers pairs were designed to obtain full-length genome sequences of IBHYM strain (Table 1). The conditions used for amplification were as follows: initial denaturation at 94°C for 5 min (the first denaturalization); 35 cycles of 94°C/1 min (denaturalization), 55°C/1 min (annealing), and 72°C/2 min (extension); followed by 10 min/72°C (final extension). Negative controls (DEPC water) were included in every set of PCR. PCR products were visualized by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. The strong bands that identified by positive amplification were purified using a QIAquick PCR Purification Kit or kit elute gel QIAquick Gel Extraction Kit (QIAGEN, Germany) and used directly for sequencing.

Computational analysis

A multiple nucleotide alignment of Vietnamese and published IBV sequences was produced using GENEDOC version 2.7 (Nicholas *et al*., 1997). A phylogenetic tree was constructed using MEGA7.0 using the neighbor joining method with 1,000 bootstrap replicas (Tamura *et al*., 2013). The IBV sequence obtained in this study were deposited in GenBank.

RESULT AND DISCUSSION

Diagnosis of IBV by RT-PCR

By using RT-PCR with a diagnostic primer pair to determine IBV, we identified a sample positive with virulent IBV. IBHYM sample was positive IBV because this RT-PCR product was right in size, only one clear band and high in quality which can be seen on electrophoresis image.

Table 1. Primers used to amplify the complete genome sequence of IBHYM.

Nucleotide position[a]	Forward primer	Reverse primer	Size (kb)
1-1645	ATGGCTTCAAGCCTAAAACAGG	CAATTCCTTTCAACACACAG	1.6
1442-3080	GTAAAGCTCAAATGTCAGTTGTG	ATTACAACGGTCAAATCACC	1.6
2893-3851	CTGATGTTCCTACAGAAGAAGTT	TGAAGGAGCACTATAGCTGAACT	1.0
3742-5195	ATTTGCAAACACTCGCGCAGAA	CCAAATACCTTATACAGTAGC	1.5
5100-6344	GGCAAGGATTCTCTTCATTTG	ATAAAGCTGCGGTGCGTTACTA	1.3
6226-7836	GAGAAATTGTTGGTTATACCCAC	CAGCACAATAGAACACAAGAC	1.6
7750-9312	GGAGTTGATGTTTGTAAACTCCTTC	GGATCATACCATTGGCTAAAG	1.6
9176-10944	TGGTTTTGGAGTAGATGTGTGC	CCTGCCTCATTCAAGAATGAGG	1.7
10815–12856	GTGTTGATCAAGCACACTGCAG	TGTCTGGTGTGTTATACCAGAAG	2.0
12412–14028	GCATAAGGGCTACAAGTCTTATG	ACCCAACACTTAGAGTCAGCCA	1.6
13929–14877	ACACACTTGCCAAACAAGGTCTT	GCACTAGCAAATTGCTGTTTATGC	1.0
14813–15650	AGACGCTTTGCTGCTGAGACAG	TACGAGGTGCCGGTAATTGAGCA	0.8
15520–16866	TAGTTGTGACATTCTGTTGGTAG	AAAGTAGTAGCTCTAGAACC	1.3
16738–19004	TCTATGCAATGTCTCAGATTGTG	CAAAGTGCATTACACGCATATTATGCGG	2.3
18403–20306	GTCAGAAGAGGACTTTATTG	TAGGCCCACCTGCTTTAAAATA	1.9
20071–22296	AACTCTTGTCCTTTAACAGG	GCATCAAGTTGAGCATAAATGTC	2.2
20959–21902	GAACCACTGGTGTTAACTCAAC	AGATAAGATCCTTAAGAGTGCC	1.0
21266–22974	GGTTCTCAGTCTCTTGAAAATCAG	ATTGACAGTTCTTCAAGGTC	1.7
22811–24906	GATGAGTTGTCAAAATGGTGG	AAGGCTCTGCTTGTCCTGCTTT	2.0
24764–25867	ACGCCAAACCAGTCGCTGGTAT	GCTAGGTGTAAGGTTGAGCATTG	1.1
25088–26426	AAACTAGGAGGGCCAAAACCAC	TCAGAGGAATGAAGTCCCAAC	1.3
26264–27672	GGTGATTCTCAAGATGGTAT	GCTCTAACTCTATACTAGCCT	1.4

[a]Primer locations are listed according to the IBHYM strain.

Sequence analysis of the whole genome of Vietnamese IBV strain

The cDNA of the IBHYM strain was converted and used as templates for coupled primer-walking PCR and sequencing to obtain the complete genome nucleotide sequence. Primers are listed in Table 1. DNA contigs were assembled to generate the complete genome sequence in MacVector 8.2. The length of the genome of IBHYM was 27,672 bp, with a (G+C) content is 10,452 nucleotide (37.77%), (A+T) content is 17,220 nucleotide (62.23%) (Accesion number in GenBank: OR631177/PP669338). The result showed that IBV genome contained much A and T. This characteristic may be one reason make genome of this virus is continuously variable.

Table 2. Sequential gene arrangement of the genome features of IBHYM strain.

Open Reading Frame (ORF)	Gene	Position	Size (nucleotide)	Products (protein)
1		533–2554	2021	leader protein p87
		2555–8752	6198	coronavirus nsp1 (HD1)
		8873-9793	921	coronavirus nsp2 (3CL-Pro)
		9794-10675	882	coronavirus nsp3 (HD2)
		10676-10924	249	coronavirus nsp4
		10925-11554	630	coronavirus nsp5
		11555-11887	333	coronavirus nsp6
	1 ab (19,893 bp)	11888-12322	435	coronavirus nsp7 (GLF)
		12323-12391	69	coronavirus nsp8
		12323-12364	2820	coronavirus nsp9
		12364-15142		(RNA-dependent RNA polymerase)
		15143-16942	1799	coronavirus nsp10 (metal-binding NTPase/helicase)
		16943-18505	1563	coronavirus nsp11
		18506-19519	1014	coronavirus nsp12
		19520-20425	906	coronavirus nsp13
2	S	20378-23881	3504	spike protein
3	3a	23881-24054	174	3a protein
	3b	24054-24245	192	3b protein
	3c	24226-24558	333	3c small virion-associated protein
4	M	24527-25207	681	membrane protein
5	5a	25561-25758	198	5a protein
	5b	25755-26003	249	5b protein
6	N	25946-27175	1230	nucleocapsid protein

The genome has the typical genetic structure of IBV strains as follows: 5′UTR-1ab-S-3a-3b-3c (E) -M-5a-5b-N-3′UTR (Adams *et al*., 2016). At this time, have no complete Vietnamese IBV genome published on NCBI.

ORF1ab is 19,893 nucleotides in size and encodes 14 non-structural proteins (including the leader protein and Nsp1–13). The remainder of the genome encodes four structural proteins, including S (Spike), E (Envelope), M (Membrane), N (Nucleocapsid) proteins, and two other non-structural proteins, including 5a, and 5b (Table 2).

Gene 2 (ORF2) has a size of 3,504 nucleotides and encodes an S protein includes 1,167 amino acid in size. The S protein is cleaved into two small units, S1 and S2, with sizes of 542 aa and 625 aa, respectively. The cleavage site on the S protein is RRFRR.

Sequence comparison

In the study, the Genedoc 2.7 program was used to compare the nucleotide and amino acid sequence of the full S gene of the IBHYM strain with those of strains from all over the world with different genotypes that were already in GenBank. The results are presented in Table 3. IBV strains in the same genotype have uniformity rates of nucleotide and amino acid gene S, reaching over 79.9% for amino acid and 87.5% for nucleotide (Table 3). However, among genotypes, this rate was low (from 70.2% to 98.7% for nucleotide and 52.6% to 97.5% for amino acid). Comparison results based on the entire S gene also showed that the IBHYM strain has higher nucleotide and amino acid similarities with strains of serotype QX-like (genotype GI-19) (87.5% to 95.0% for nucleotide and 80.2% to 91.3% for amino acid) and the lowest similarity to strains of serotype TC07-like (genotype GVI-1) (70.4% to 70.5% for nucleotide and 53.8% to 56.2% for amino acid). The variation of both nucleotides and amino acids among IBV strains was on the S1 gene. Previous studies have shown that the S2 subunit is highly conserved and mutations are mainly in the S1 gene (Cavanagh and Davis, 1986; Ismail *et al*., 2001).

Phylogenetic analysis of IBV genotypes

Based on the full S nucleotide sequences, a phylogenetic tree was constructed to examine the relationships between the IBHYM Vietnamese strain and 30 other IBV strains of representative genotypes by MEGA 7.0 (Figure 1).

Based on the results of phylogenetic analysis, IBV isolates from different genotypes were put into several genetic groups. These groups were Massachusetts (GI-1), Q1-like (GI-16), 4/91-like (GI-13), Taiwan-type (GI-7), QX-like (GI-19), LDT3-type, and TC07-like (GVI-1). In which the strain IBHYM identified in Vietnam is classified as belonging to the same serotype as QX-like, respectively GI-19 genotype.

Recently, due to how complicated IBV is, some scientists have come up with a new classification system (genogrouping) based on the sequence of hypervariable regions on the S1 gene of nearly 1,300 IBV strains (Valastro *et al*., 2016). The results have been systematized and classified IBV into 7 large groups: GI, GII, GIII, GIV, GV, GVI, and GVII, of which, up to now, GI is divided into 29 subgroups (GI-1 to GI-29) (Hou *et al*., 2020; Ghetas *et al*., 2021). Currently, in China, there are at least four main IBV lines,

including QX-type (GI-19), Taiwan I (GI-7), TC07-2 (GVI-1) and 793B-type (GI-13), which exist in chicken farms, significantly increasing the difficulties in disease prevention and control. In Vietnam, beside four above main IBV strains, Q1 like strains (GI-16) are existed, too.

The results of the investigation also revealed that the field strain IBHYM has low similarity with the Massachusetts vaccine (strain H120) that is currently in use (81.7% for nucleotide and 69.2% for amino acid) (Table 3). The differences exist in many regions of genome but the most different region is S gene, especially S1. This data is similar to other research that shows an increase in viral isolates from vaccinated chickens (Zanaty *et al*., 2016; Abdel-Sabour *et al*., 2017; Abozeid *et al*., 2017). The causes of vaccination failure can be attributed to the fact that field infections have a high rate of change in the S antigen gene region, and even a slight alteration in the amino acid sequence of this protein can have a major impact on the vaccine's performance and may result in the formation of new genotypes and/or serotypes that are antigenically unique from currently available classical vaccine strains (Zanaty *et al*., 2016; Abdel-Sabour *et al*., 2017; Abozeid *et al*., 2017). Studies have shown that even a 5% mutation in the S1 gene can alter vaccination protection (Wang and Huang., 2000). As a result, molecular epidemiology monitoring and surveillance are critical not only for elucidating the molecular properties of circulating viral strains but also for assisting in the selection of appropriate vaccination strains to achieve a high preventative impact.

Table 3. Percentage (%) of nucleotide identity (above the line) and amino acid identity (below the line) in the S gene of IBV strains.

	IBHYM	QX-like			HN08-type		4/91-type		Q1-like		TWI		Mass-type		LDT3-type		TC07-2-like	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		94.9	95.0	87.5	94.8	91.4	86.0	90.8	80.7	82.6	85.0	84.3	81.7	81.7	91.8	88.8	70.5	70.4
2	91.3		96.1	88.2	98.7	92.2	87.4	91.2	81.2	83.0	84.0	83.3	81.7	81.7	92.4	88.6	71.5	71.3
3	90.7	92.2		88.1	95.8	93.1	86.1	90.9	81.7	83.5	84.7	83.9	81.7	81.7	92.6	89.1	71.0	71.0
4	80.2	79.9	80.7		88.1	87.1	82.1	89.5	80.4	82.2	83.6	80.8	81.6	81.6	90.4	91.3	72.0	71.7
5	90.9	97.5	92.1	79.5		91.8	87.5	91.2	81.1	82.9	83.8	82.9	81.6	81.7	92.4	88.5	71.1	70.9
6	84.3	85.7	86.7	78.0	84.9		82.4	86.4	81.1	82.7	85.8	84.8	83.4	83.4	90.4	90.2	71.2	71.0
7	77.1	78.3	76.1	68.8	78.4	70.2		91.2	83.7	82.3	82.1	81.6	83.6	83.6	85.4	82.1	71.2	71.0
8	86.2	85.1	85.3	81.9	84.8	77.1	84.7		81.1	82.9	83.9	82.7	82.0	82.0	90.7	88.5	71.5	71.2
9	67.8	67.5	69.1	67.3	67.5	68.3	72.7	68.0		96.3	81.4	81.6	82.1	82.1	80.7	80.1	70.5	70.4
10	71.5	71.1	72.8	70.8	71.3	71.5	70.4	71.9	92.6		83.5	83.6	80.4	80.4	82.6	82.0	70.4	70.2
11	75.5	74.0	74.7	72.8	73.6	77.6	70.9	73.4	69.9	74.0		92.4	84.5	84.6	84.3	84.6	71.0	71.1
12	74.6	73.2	73.9	73.0	72.4	76.6	69.2	71.3	68.8	73.0	87.3		82.8	82.8	83.8	84.0	71.3	71.2
13	69.2	67.5	68.4	69.0	67.4	71.6	71.3	68.6	71.5	68.0	74.7	71.8		99.9	82.8	82.9	71.1	70.9
14	69.2	67.5	68.4	69.0	67.4	71.6	71.3	68.6	71.5	68.0	74.7	71.8	100.0		82.8	83.0	71.2	70.9
15	86.4	87.1	87.4	83.3	86.8	83.9	74.4	83.7	67.9	72.1	74.6	73.9	70.0	70.0		94.6	71.5	71.2
16	81.8	80.6	81.4	85.1	80.3	83.6	69.2	80.3	66.8	70.4	75.3	74.4	70.5	70.5	90.6		71.5	71.2
17	53.8	54.4	54.3	55.7	53.9	54.6	53.7	54.1	53.0	52.9	54.3	54.7	54.7	54.7	55.6	56.2		98.3
18	53.8	54.4	54.4	55.5	53.9	54.4	54.0	54.2	53.0	52.6	54.4	54.6	54.4	54.4	55.5	55.7	96.9	

1.IBHYM, 2.Ck/CH/LGD/090907(KP118894), 3.ck/CH/LSD/111235(118886), 4.SAIBK2(KU317090), 5.Ck/CH/LGD/120723(KP013541), 6.YN(JF893452), 7.4/91 vaccine(KF377577), 8.Ck/CH/LZJ/111113(JX195176), 9.VNUA3(KY992863), 10.ck/CH/LDL/97I(JX195177) 11.ck/CH/LHB/100801(JF330893), 12.3575/08(KX266757), 13.H120(MN548287), 14.ck/CH/LDL/101212(JF828981), 15. CK/CH/2010/JT/1(KU361187), 16. ck/CH/LGX/111119(KX640829), 17. VNUA11(KY992865), 18. CK/CH/SD09/005(KF668605).

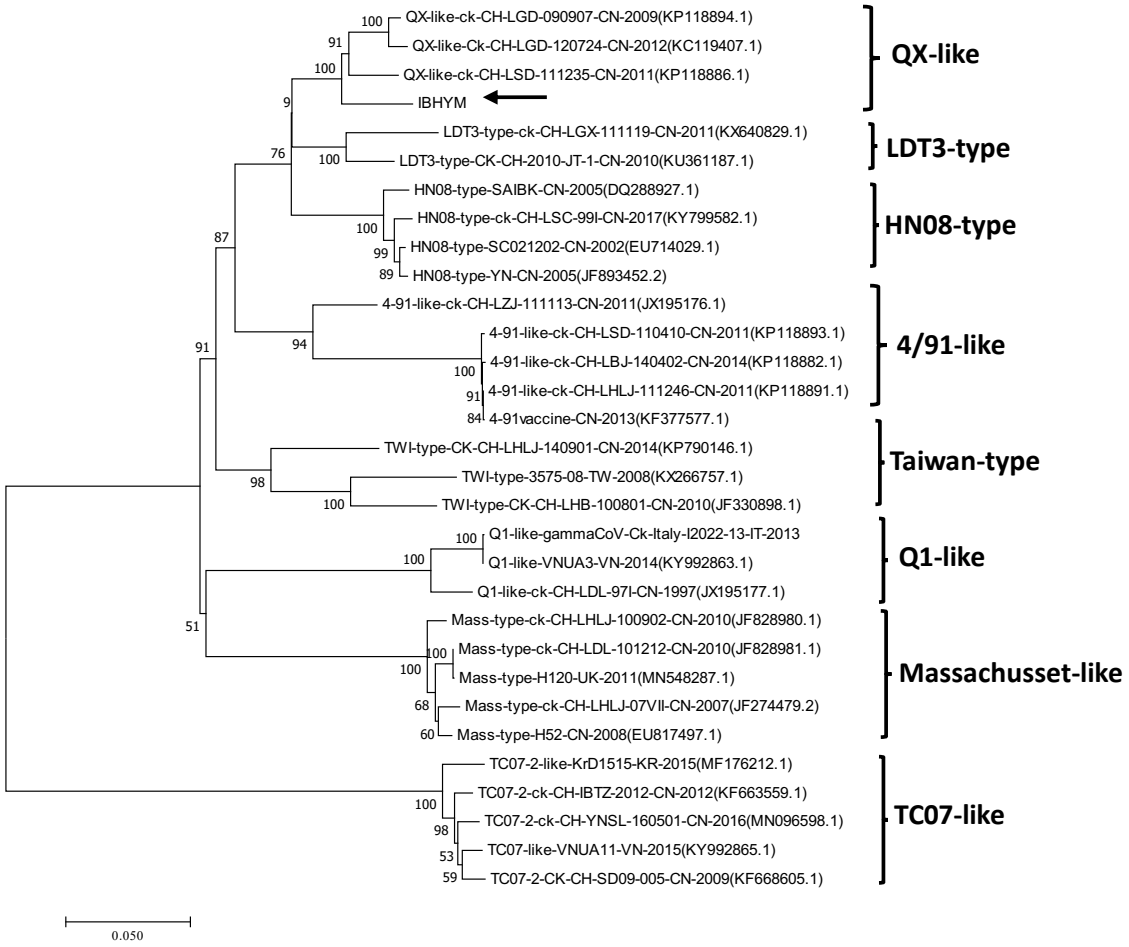

Figure 1. Phylogenetic tree showing the relationships between the IBHYM Vietnamese strain and 30 IBV strains of different genotypes based on analysis of the full S nucleotide sequences. The phylogenetic tree was constructed with the MEGA 7.0 package using the neighbor-joining method (Tamura *et al.*, 2013) with bootstrap values of 1000 replicas (shown at each branch). The scale bar at the bottom indicates the number of nucleotide substitutions per site. The isolate in this study is indicated by an arrow. The accession numbers are given at the end of each sequence, where applicable. International country codes (https://countrycode.org/) are in brackets, e.g., VN, Vietnam; CN, China; TW, Taiwan; KR, South Korea; CA, Canada; IT, Italy.

CONCLUSION

The full genome of the IBV strain causing infectious bronchitis in chickens in Hung Yen province in 2021 was sequenced. The causal IBV strain is QX-like and belongs to the GI-19 genotype. QX-like has been recognized as a prevalent pathogen in China and Vietnam nowadays. The results we obtained will be helpful in understanding the molecular characterization of IBV in Vietnam. There has been no complete Vietnamese IBV genome published on NCBI before.

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