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Phylogenetic analysis of the S1 glycoprotein gene of infectious bronchitis viruses isolated in China during 2009–2010

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Abstract As part of our ongoing surveillance program, 40 field strains of avian infectious bronchitis virus (IBV) were isolated from dead or diseased chicken flocks in different areas of China between 2009 and 2010. S1 glycoprotein genes of these strains were sequenced and analyzed with 38 strains published in GenBank. S1 genes of these isolated strains and the vaccine strains showed nucleotide homologies ranging from 65.2 to 82% and amino acid homologies ranging from 58.4 to 81.9%. Meanwhile, Chinese IBV strains isolated in this study, which were mainly nephropathogenic, could be separated into six variant lineages (CH I–CH VI), and current vaccine strains used in China formed Mass variant lineage that is evolutionarily distant from Chinese isolates. Moreover, CK/CH/GD/NC10, CK/CH/GD/KP10, and our previous isolates TC07-2 formed the CH VI lineage, showing larger evolutionary distances from other strains. Taken together, these findings suggested that various variant lineages were co-circulating in China now, and appeared to be continuously evolving, alternative indigenous vaccines indeed need for effective control of IB in China.

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Infectious bronchitis (IB), an economically important disease to poultry industry, is caused by the infectious bronchitis virus (IBV) which belongs to the Coronaviridae family. As a highly infectious and contagious pathogen of chickens, IBV not only causes upper respiratory disease, but also replicates in kidney and oviduct [1, 2]. IBV genome consists of a positive-stranded RNA encoding four structural proteins, envelope (E) glycoprotein, integral membrane (M) glycoprotein, phosphorylated nucleocapsid (N) protein, and spike (S) glycoprotein [3, 4]. S glycoprotein is posttranslationally cleaved into S1 and S2 by cellular proteases. S1 forms the tip of the spike, and S2 anchors the S1 to the viral membrane. S1 as the major inducer of protective immunity is the main protein responsible for attachment and subsequent infection of target cells and tissue tropism. Furthermore, S1 induced the production of neutralizing and hemagglutinating antibodies [5–8]. Several amino acids differences of S1 can result in a new serotype strain, and lead to poor cross-protection, but the mutations of the S1 gene are not always associated with the new antigenic variants [9–11].

Though commercial chickens have been vaccinated, IBV is still the main cause of chicken respiratory diseases as indicated by many field reports. Under the pressure of immunity induced by vaccine strains, the mutations of S1 are believed to endow variant strains with selective advantages. We have reported that most field strains were phylogenic distant from vaccine strains [12]. There have been constant and frequent outbreaks of IB in commercial flocks in China in the past 2 years, especially in winter and spring.

In this research, as part of our ongoing surveillance program, we have isolated 40 IBV field strains from broilers with respiratory problems and nephrosis–nephritis or breeders showing egg drop in 13 provinces of China between 2009 and 2010, and the S1-coding region of the isolated 40 IBV field strains was sequenced. All of the flocks were vaccinated before 10 days of age with a live vaccine of Mass serotype (H120 or H52 strain). Viruses were propagated in 9- to 11-day-old embryonated specific pathogen-free (SPF) chicken eggs. The allantoic fluids were collected 48 h post-inoculation for RNA extraction, and viruses were identified as IBV by RT-PCR for the N protein gene. The details of these isolates were summarized in Table 1.

Viral RNA was extracted using the Axy Prep™ Body Fluid Viral DNA/RNA Miniprep Kit (AXYGEN). A pair of primers for amplifying S1 coding region was designed using Primer Premier 5.0 software based on alignment of GenBank sequences of several known IBV strains from China. The sense primer: 5′-TTG AAA ACT GAA CAA AAG ACC G-3′, and the anti-sense primer: 5′-TAC AAA ACC TGC CAT AAC TAA CAT-3′. The anticipated amplification segment is about 1760 bp encompassing S1 coding region including the protease cleavage motif. S1 gene was amplified using PrimeScript One Step RT-PCR Kit Ver.2 (TAKARA, Japan). PCR products were purified using Axy Prep™ DNA Gel Extraction Kit (AXYGEN) and then cloned into PMD19-T vector (TAKARA). For each gene, three clones were chosen for sequencing by the dideoxy chain termination procedure using the automated ABI Prism 3730, Genetic Analyzer. The nucleotide sequences of the S1 genes reported here were deposited in GenBank, and the accession numbers are shown in Table 1.

The amino acid sequences were deduced and sequence alignments were performed by the DNAStar software (DNASTAR Inc., USA). As shown in Table 1, S1 gene of the isolated strains contain 1599, 1611, 1614, 1617, 1620, 1626, 1632, 1638 nucleotides, and 533, 537, 538, 539, 540, 542, 544, 546 amino acids, respectively. Only the strains of CK/CH/GD/NC10 and CK/CH/GD/KP10 contain 1638 nucleotides and 546 amino acids. The homology of the nucleotide and deduced amino acid sequences of S1 genes (data not shown) among the 40 isolated strains were 65.4–99.9 and 58.4–99.6%. When compared with the widely used vaccine strain H120, the homology of the nucleotide and deduced amino acid sequences were 65.2–82 and 58.4–81.9%.

Nucleotide sequences of S1 coding region of 40 isolates and 38 reference strains was aligned by Clustal X. Then a neighbor-joining phylogenetic tree was constructed by MEGA 4. The bootstrap values were determined from 1,000 replicates of the original data. It was clearly shown that all these isolated strains were separated into six variant lineages (CH I–CH VI) (Fig. 1). Group CH I consisted of 15 isolated strains, designated as QX like variant lineage with the inclusion of Chinese QXIBV strain and Netherlandish QX-like strain L-1148; 10 isolates and 6 reference strains formed group CH II, and 4/91 strain was clustered into this big branch; group CH III of 4 isolates; group CH IV of 6 isolated and 5 Chinese reference strains, and it is interested that Pigeon coronavirus PSH050513 strain was closely related to those isolates in this group; CK/CH/SC/DY09, CK/CH/SC/ZJ10-1, CK/CH/HuN/NX09, along with Taiwanese TW-I variant lineage strain 3468/07 were grouped into the group CH V; CK/CH/GD/NC10, CK/CH/GD/KP10, and our previous isolates TC07-2 formed the group CH VI, which showed a distant relationship with other strains. Most foreign reference IBV strains showed evolutionary distances to Chinese IBV isolates according to the branch distribution of the phylogenetic tree. Different variant lineages of IBV strains were co-circulating in chicken flocks of same area, for example, both the isolates CK/CH/HB/HC09-2 and CK/CH/HB/HC09-1 were isolated in the same area and same time of Hubei province, but were clustered into variant lineage CH I and CH II, respectively, and showed large evolutionary distance. The strains from different parts could show high amino acid sequence similarities, CK/CH/SC/ZJ10-1 and CK/CH/HuN/NX09, which were isolated in Sichuan and Hunan province in 2009, respectively, clustered into one lineage. The results strongly suggest that IBV epidemiology is very complicated in China. There are also many genotypes co-circulating in Europe, USA and other parts of the world [13–16].

The most of isolates were grouped into variant lineages CH I, CH II, CH III, and CH IV, forming a large evolutionary branch, and only two isolates were classified into CH VI. CH V lineage comprised of Chinese strains and Taiwanese strains, showed the evolution process in Taiwan Island is not isolated and had some relationship with Chinese Mainland. Finally, Chinese isolates in this study did not show a closer relationship with H120, H52, Mass41 or W93 that are widely used vaccine strains in China. It may explain the cause of the immune failure in the clinic.

It can be seen that many isolates (15 of 40) cluster with QX IBV to form CH I. According to our clinical observation, there were no proventriculitis-associated isolates in our study. In the 15 QX-like isolates, 9 caused typical nephropathogenic, 4 showed typical respiratory clinical signs. In addition, proventriculitis-associated strain ZJ971 was clustered into Mass group. The relationship between the genetic characteristics, pathogenicity and tissue tropism is still unknown.

CK/CH/SC/DY09, CK/CH/SC/ZJ10-1, and CK/CH/HuN/NX09 showed amino acid similarities of 96.7–97% with Taiwanese strain 3468/07(TW-I), and formed CH V

Table 1 IBV strains isolated from flocks in different provinces of China

IBV/isolates	Province[a]	Year of isolation	Major clinical signs	Production type	Length (nt/aa)[b]	Accession number
CK/CH/GD/HY09	Guangdong	2009	Nephritis	Broiler	1626/542	HQ018887
CK/CH/GD/LZ09	Guangdong	2009	Nephritis	Breeder	1620/540	HQ018896
CK/CH/GD/XX09	Guangdong	2009	Nephritis	Broiler	1620/540	HQ018907
CK/CH/GD/YN09-1	Guangdong	2009	Nephritis	Broiler	1626/542	HQ018908
CK/CH/GD/YN09-2	Guangdong	2009	Nephritis	Broiler	1617/539	HQ018909
CK/CH/GX/NN09	Guangxi	2009	Nephritis	Broiler	1620/540	HQ018900
CK/CH/GX/YL09-1	Guangxi	2009	Nephritis	Broiler	1620/540	HQ018904
CK/CH/GX/YL09-2	Guangxi	2009	Respiratory	Broiler	1620/540	HQ018905
CK/CH/HB/HC09-1	Hubei	2009	Respiratory	Broiler	1629/543	HQ018883
CK/CH/HB/HC09-2	Hubei	2009	Nephritis	Broiler	1620/540	HQ018884
CK/CH/HB/JL09	Hubei	2009	Nephritis	Broiler	1629/543	HQ018892
CK/CH/HN/HN09	Hainan	2009	Egg drop	Breeder	1620/540	HQ018886
CK/CH/HuN/NX09	Hunan	2009	Nephritis	Broiler	1620/540	HQ018899
CK/CH/JS/LYG09	Jiangsu	2009	Nephritis	Broiler	1617/539	HQ018895
CK/CH/JS/NJ09	Jiangsu	2009	Nephritis	Broiler	1620/540	HQ018902
CK/CH/JX/JA09-1	Jiangxi	2009	Nephritis	Broiler	1632/544	HQ018890
CK/CH/JX/JA09-2	Jiangxi	2009	Nephritis	Broiler	1632/544	HQ018891
CK/CH/SC/DY09	Sichuan	2009	Nephritis	Broiler	1620/540	HQ018882
CK/CH/SC/MS09	Sichuan	2009	Nephritis	Broiler	1626/542	HQ018897
CK/CH/ZJ/HZ09	Zhejiang	2009	Respiratory	Broiler	1620/540	HQ018888
CK/CH/AH/HF10	Anhui	2010	Nephritis	Broiler	1620/540	HQ018885
CK/CH/FJ/PT10	Fujian	2010	Nephritis	Broiler	1626/542	HQ018898
CK/CH/GD/KP10	Guangdong	2010	Egg drop	Breeder	1638/546	HQ018919
CK/CH/GD/LY10	Guangdong	2010	Nephritis	Broiler	1620/540	HQ018894
CK/CH/GD/NC10	Guangdong	2010	NA	Broiler	1638/546	HQ018903
CK/CH/GD/XX10	Guangdong	2010	Nephritis	Broiler	1620/540	HQ018906
CK/CH/GD/ZX10	Guangdong	2010	Nephritis	Broiler	1614/538	HQ018921
CK/CH/GX/GL10	Guangxi	2010	Respiratory	Broiler	1620/540	HQ018910
CK/CH/GX/LC10	Guangxi	2010	Respiratory	Broiler	1617/539	HQ018911
CK/CH/GX/YL10-1	Guangxi	2010	Nephritis	Broiler	1626/542	HQ018912
CK/CH/GX/YL10-2	Guangxi	2010	Nephritis	Broiler	1620/540	HQ018913
CK/CH/HeB/CZ10	Hebei	2010	Nephritis	Broiler	1599/533	HQ018916
CK/CH/HN/HN10	Hainan	2010	Nephritis	Broiler	1626/542	HQ018915
CK/CH/JS/JL10	Jiangsu	2010	NA	Broiler	1620/540	HQ018893
CK/CH/JS/NJ10	Jiangsu	2010	NA	Broiler	1620/540	HQ018901
CK/CH/SC/MS10	Sichuan	2010	Nephritis	Broiler	1620/540	HQ018914
CK/CH/SC/ZJ10-1	Sichuan	2010	NA	Broiler	1620/540	HQ018918
CK/CH/SC/ZJ10-2	Sichuan	2010	Respiratory	Broiler	1620/540	HQ018920
CK/CH/TJ/NH10	Tianjin	2010	Egg drop	Breeder	1611/537	HQ018917
CK/CH/ZJ/HZ10	Zhejiang	2010	Nephritis	Broiler	1620/540	HQ018889

NA not available

[a] Province where the viruses were isolated

[b] Lengths of nucleotides and deduced amino acids of S1 glycoprotein gene

lineage, implying that the evolution process in Taiwan is not an isolated one. Remarkably, one IBV strain of TW-type CK/CH/SCMY/10I was also isolated in Sichuan recently [17]. But previous research reveals that IBV strains from Taiwan formed a unique genotype that was separated from the Chinese genotypes [12, 18]. Taiwan is a

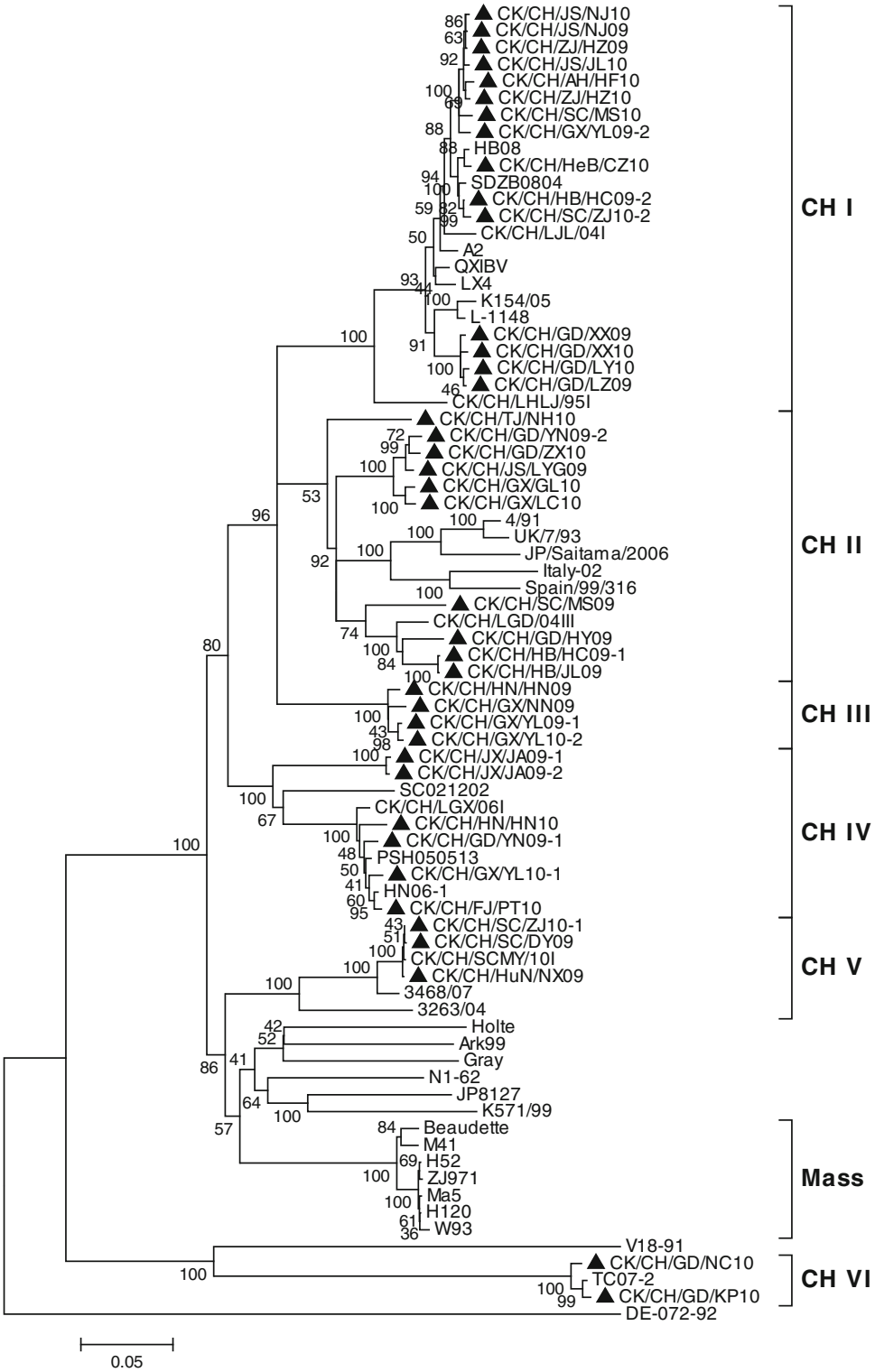

Fig. 1 Phylogenetic tree constructed by neighbor-joining method with pairwise deletion and set bootstrap 1,000. (MEGA 4.0). S1 nucleotide sequences of the 40 isolated and 38 published IBV strains were included for the phylogenetic analysis, where the 40 isolated IBV strains are marked with *ante-black triangle*

geographically separate island from China by 100 miles of water. If there is no illegal trafficking or unapproved attenuated vaccine used in the field, considering the trading of poultry is forbidden, Taiwan researchers speculated that migrating birds provided the genetic sources of IBV variants in Taiwan [19]. Peafowl and teal may be the hosts as carriers of IBV, and have potential to transmit viruses to susceptible chicken populations [20]. In this study, a coronavirus from pigeons (PSH050513) was grouped into the CH IV. It also had high degree of amino acid sequence identity of 94.6–99.1% between CK/CH/HN/HN10, CK/CH/GD/YN09-1, CK/CH/GX/YL10-1, CK/CH/FJ/PT10,

and PSH050513, which were located in different provinces. PSH050513 has been tentatively identified as a novel member of group 3 coronaviruses that have close genetic relationship with IBV strains [21, 22]. May be migrating birds spread the virus.

Three strains (CK/CH/GD/NC10, CK/CH/GD/KP10, and TC07-2) were clustered in a distinct new phylogenetic group CH VI, which were well separated from the other strains and shared only 57–62% amino acid identity with the S1 proteins of other group strains. These strains also had a 4 amino acids insertion (Gln-Lys-Glu-Pro) at the region of 283–284 same as TC07-2 strain (data not shown) [12]. It has been reported that S1 amino acids of different IBV serotypes usually differ by 20–25% [23]. This indicates that the strains of CH VI lineage are a different serotype than the vaccine strains and the other types of IBV isolates examined. In this cluster, TC07-2 was isolated in Guangdong province in 2007 [12], the other two isolates (CK/CH/GD/NC10 and CK/CH/GD/KP10) from 2010 were isolated in west of Guangdong province, where is far from the place of TC07-2 isolated. This novel IBV was also isolated in Japan recently (designated JP-IV), and the introduction route of this novel IBV into Japan and its prevalence are unknown [13], indicating these "novel" strains may spread to many chicken flocks in recent years. Because the CH VI strains continue to persist in the field and have a unique sequence compared with other strains of IBV, it is an excellent choice for studying genetic drift and recombination, may be strains of CH VI has undergone recombination event as well as extensive antigenic variation like DE072 strain [24], but need further research to testify.

In conclusion, the present study demonstrated that the various variant lineage IBV strains were co-circulating in commercial flocks in China. The data also indicated that phylogenic distant IBV strains continued to emerge, and there were no predominant IBV strains circulating in the fields in China yet as we reported earlier [12]. Interestingly, two field isolates were classified into the new phylogenetic group CH VI; what impact they might exert on IBV's spread and circulation needs to be monitored. This paper is a periodic report on our ongoing surveillance program, and it manifests the importance of continuing surveillance of IBV strains.

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