Research paper

Phylogenetic and antigenic analysis of avian infectious bronchitis virus in southwestern China, 2012–2016

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ABSTRACT

The aim of this study was to decipher the molecular epidemiological and antigenic characteristics of infectious bronchitis virus strains (IBVs) isolated in recent years in southwestern China. A total of 24 field strains were isolated from diseased chickens between 2012 and 2016. Phylogenetic analysis based on S1 nucleotide sequences showed that 16 of the 24 isolates were clustered into four distinct genotypes: QX (37.5%), TW (16.7%, TWI and TWII), Mass (8.3%), and J2 (4.2%). The QX genotype was still the prevalent genotype in southwestern China. Recombination analysis of the S1 subunit gene showed that eight of the 24 field strains were recombinant variants that originated from field strains and vaccine strains. A new potential recombination hotspot [ATTTT(T/A)] was identified, implying that recombination events may become more and more common. The antigenicity of ten IBVs, including seven field strains and commonly used vaccine strains, were assayed with a viral cross-neutralization assay in chicken embryonated kidney cells (CEK). The results showed that the ten IBVs could be divided into four serotypes (Massachusetts, 793B, Sczy3, and SCYB). Sczy3 and 793B were the predominant serotypes. Six of the seven field isolates (all except for cK/CH/SCYB/140913) cross-reacted well with anti-sera against other field strains. In conclusion, the genetic and antigenic features of IBVs from southwestern China in recent years have changed when compared to the previous reports. The results could provide a reference for vaccine development and the prevention of infectious bronchitis in southwestern China.

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1. Introduction

Infectious bronchitis (IB) is a highly contagious disease in chickens that causes significant economic losses to the worldwide poultry industry (Colvero et al., 2015). The etiologic agent of IB is the infectious bronchitis virus (IBV), a member of the Coronaviridae family, in the subfamily coronaviridae and genus gamma-coronavirus. The IBV genome is 27.6 kb and encodes at least four structural proteins, including the spike glycoprotein (S), membrane protein (M), small membrane protein (E), and nucleocapsid protein (N) (Ujike and Taguchi, 2015). The major immunogen of IBV is the S1 subunit protein, which contains epitopes that can induce the production of specific neutralizing antibodies and the hemagglutination inhibition antibody. IBVs from different serotypes usually exhibit poor cross-protection (Li et al., 2012). Due to the incomplete proofreading mechanism of the RNA polymerase and

the gene recombination during genome replication, IBV genomes are constantly evolving, and new IBV variant strains are always arising (Baker and Lai, 1990; Lai, 1992).

Since the early 1980s, IBV has been diagnosed in China by viral isolation. Although the wide use of vaccine strains, such as H120, M41, 28/86, 4/91, and ma5, has successfully prevented IB epidemics on most farms, immune failure is still reported frequently as the result of infections with strains that differ serologically from the vaccine strains. Therefore, continuing analysis of the genetic evolution and antigenic relatedness among field isolates and vaccine strains may provide critical insight for vaccine strain selection and vaccine development. Our previous study revealed that isolates obtained between 2008 and 2009 from the Sichuan province belonged mainly to a group of QX-like strains (79% QX-type; 5% TWI-type) (Zou et al., 2010). In a later report from other researchers, QX-type and TWI-type IBVs accounted for 37% and 37%, respectively, in Sichuan area during 2011–2012 (Zhang et al., 2016). While in Southern China, picture was quite different, cK/CH/LSC/99I-type was the predominant genotype and no QX-type strains were isolated during 2013 (Mo et al., 2013). So the genetic character of IBVs from China varied according to time and regions. For the antigenic features of IBVs isolated in recent years from China, less attention was given. A report showed that 28 IBVs from Guangxi of China in 2009–

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2011 could be divided into 6 serotypes (I-VI), but most of the isolates (24/28) was QX-genotype, IBVs from the QX-genotype may belong to different serotypes, and the serotype of IBVs varied according to time and regions (Qin et al., 2014). Another report showed the serotype of TWI-type strains were different from Mass-type in Taiwan in 2000 (Wang and Huang, 2000). As the genetic and antigenic character of IBVs varied according to time and regions, and there is no official report on that of the IBVs from southwestern China in recent years, the molecular and antigenic characteristics of IBVs from southwestern China were not clear. The aim of this study was to decipher the genetic and antigenic characteristics of IBV strains circulating in commercial flocks in southwestern China in recent years.

2. Materials and methods

2.1. Eggs and virus

Specific pathogen-free (SPF) chicken embryos were obtained from Beijing Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China). M41 and H120 strains were obtained from the China Institute of Veterinary Drug Control (Beijing, China). The 4/91 vaccine was supplied by Internet International B.V. (Boxmeer, NL).

2.2. Viral isolation

Throughout 2012–2016, kidney, lung, and trachea samples were collected from broiler or layer chickens suspected of IB infection in southwestern China (Table1). Samples were homogenized in phosphate-buffered saline (PBS) containing 200 μg/mL penicillin and 100 μg/mL streptomycin in a ratio of 1:5–10. After filter sterilizing with a 0.22 μm filter membrane, 0.2 mL sample was inoculated into the allantoic cavity of 9- to 11-day-old SPF embryos. The embryos were incubated at 37 °C and examined twice daily for their viability. The allantoic fluids were harvested after 36 h incubation, and three blind passages were conducted. The presence of IBV was verified by reverse transcription-polymerase chain reaction (RT-PCR) of the N gene (Zou et al., 2010). The existence of other five pathogens, H9 subtype Avian influenza virus (H9 AIV), Newcastle disease virus (NDV), Marek's disease virus (MDV), bacteria and Coccidiosis in those samples were verified by following the methods of other reports (Abu-Akkada and Awad, 2012; Chen et al., 2012; Li et al., 2010; Rui et al., 2010; Tian et al., 2011).

2.3. Phylogenetic and recombination analysis of the S1 genes

Total RNA was extracted from IBV-infected allantoic fluid with RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions and dissolved in 40 μL sterile diethylpyrocarbonate (DEPC)-treated water before being stored at −70 °C for further use. For the reverse transcription (RT) reaction, 5 μL of template RNA, 2 μL of 5× RT Mix, and 3 μL of RNase-free water were added and mixed. The reaction mixture was incubated at 37 °C for 15 min and then at 85 °C for 1 min. PCR amplification and cloning of the S1 gene was performed as the previous report (Zou et al., 2010). The recombinant plasmids containing the target gene were sequenced by Shanghai Sanggong Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Nucleotide sequences of the S1 gene obtained from the IBV isolates were aligned using the Editseq program in the Lasergene package (DNASTAR Inc., Madison, WI, USA) and compared to the sequences of 53 other reference IBVs using the MegAlign program in the same package. For the 53 reference IBVs, 43 strains were isolated from China, 2 strains were isolated from the USA, 2 strains were from Japan, and the other 6 strains were vaccine strains. A phylogenetic tree of the S1 gene was created using the neighbor-joining method in MEGA version 7.0.14. Bootstrap values were determined from 1000 replicates of the original data.

The S1 subunit sequences of 24 IBV field strains and 53 reference strains were aligned by MegAlign, and putative recombinant strains were selected by sequence homology analysis. In order to identify the assumed parent sequences, the S1 subunit sequences of suspected recombinant isolates were blasted against the GenBank database of the National Center for Biotechnology Information (NCBI). Recombination analysis of the selected sequences was conducted with the aid of Recombination detection program (RDP 4.72) and SimPlot version 3.5.1 software. Potential recombination events were identified using the RDP, Maxchi, and GENECONV methods in RDP 4.72 to identify putative parental sequences with significance set at *P* values <0.05 and the sliding window size set as 30 bp. Putative potential recombination events were further identified using the SimPlot version 3.5.1. Nucleotide identities were calculated using the Kimura 2-parameter method with a transition-transversion ratio of 2 in each window of 200 bp, and the window was successively extended in 20-bp increments.

2.4. Viral cross-neutralization test

For the preparation of antisera against the ten IBVs, 8-week old rabbits ($n = 4$) were immunized with purified 10^4 EID$_{50}$ IBVs subcutaneously mixed with an equal volume of complete Freund's adjuvant (Sigma, Missouri, USA) for the first injection, and with the same antigen emulsified in freund's incomplete adjuvant for the following two booster injections (two-week interval). Rabbits were held in separate biosafety level 2 (BSL2) isolators in the Laboratory Animal center of Sichuan Agricultural University (Ya'an, Sichuan, China) with a libitum access to feed and water and maintained under uniform standard management conditions. Approval for these animal studies was obtained from the Sichuan provincial Laboratory Animal Management Committee [Permit Number: XYYK(Sichuan) 2014-187] and the Ethics and Animal Welfare Committee of Sichuan Agricultural University. Antisera from vaccinated animals were collected at 12 days after the final immunization and stored at −20 °C.

To determine the antigenic relatedness between the field IBV isolates and the vaccine viral strains, double-direction viral cross-neutralization (VN) tests were performed in chicken embryo kidney (CEK) cells using constant viral titers and diluted serum. The tested strains came from six different genotypes and included seven IBV field isolates (Sczy3, cK/CH/SCDY/141030, cK/CH/SCLS/140104, cK/CH/CQKX/150203, cK/CH/SCYB/140913, cK/CH/SCMY/10I, cK/CH/SCYB/141102) and the three most commonly used vaccine viral strains (H120, M41, and 4/91).

Before VN testing, IBV strains were adapted to CEK cells by serial passaging. Briefly, allantoic fluid containing the IBV strain was propagated in monolayer primary CEK cells prepared from 18- to 20-day-old chicken embryos. Infected CEK cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 2% fetal bovine serum (Zhejiang Tian-hang Biological Technology Stock Co., Ltd., Zhejiang, China) and incubated at 37 °C with 5% CO$_2$. The supernatant was harvested 40 h post-inoculation and passaged blindly in CEK cells until a characteristic cytopathic effect (CPE), such as syncytia, was observed. Determination of the TCID$_{50}$ of the CEK-adapted IBVs in CEK cells was conducted per the method of Reed and Muench (1938).

For the VN test, equal volumes of 100 TCID$_{50}$ of the CEK-adapted IBVs and serial two-fold dilutions of antisera were mixed and kept at 37 °C for 1 h. Next, 0.4 mL of the virus–antisera mixture was then transferred to CEK cell cultures in 24-well plates (6 wells for each dilution). The plates were incubated for 72 h, and the 50% end-point neutralizing titers were calculated by the method of Reed and Muench (1938). Negative rabbit serum was also incubated with 100 TCID$_{50}$ of IBV to calculate its non-specific neutralizing titer to IBVs, and this neutralizing titer was used as a background value for further analysis.

The VN end-point titers were used to calculate the antigenic relatedness values (ARV, r) by the method of Archetti & Horsfall

(1950): $AVR(r) = \sqrt{r1 \times r2} \times 100\%$, where r1 represents the ratio of the heterologous neutralizing titer of virus 1 to the homologous titer of virus 1, and r2 represents the ratio of the heterologous titer of virus 2 to the homologous titer of virus 2. Isolates where r < 50% were considered to be antigenically unrelated, isolates with 50% ≤ r < 100% were considered to be antigenically related, and isolates with r ≥ 100% were considered to be antigenically identical.

3. Results

3.1. Viral isolation

A total 86 clinical samples, including trachea, lung, and kidney samples, were collected from dead or diseased chickens displaying respiratory symptoms and/or nephritis from chicken flocks located in southwestern China. This included the Si-chuan, Yun-nan, Gui-zhou, and Chong-qing areas. From these, 24 IBV strains were isolated. Typical signs of IBV, including embryo dwarfing and death, were observed during the passaging of samples through embryos. RT-PCRs of the clinical samples showed that only a few samples exhibited co-infection of the H9 AIV (2/24, 8.3%) or the NDV (1/24, 4.2%). Bacterial isolation showed that *E. coli* and *Salmonella* were often found in the clinical samples (6/24, 25.0%). The case histories of local strains are listed in Table 1.

3.2. Phylogenetic analysis of S1 gene

S1 gene sequences from the 24 IBV isolates were determined and submitted to GenBank under the accession numbers KU364603–364620, KU516832, KX129907–129909 and KX344067-344068. The full-length S1 subunit gene open reading frame (ORF) ranged from 1605 to 1626 bp. Phylogenetic analysis based on S1 nucleotide sequences of the 24 wild strains showed that 16 of the 24 isolates could be grouped into four genotypes: QX, J2, TW (TWI and TWII), and Mass. Nine field isolates (cK/CH/SCDY/141030, cK/CH/GZGY/150203, cK/CH/SCMS/140618, cK/CH/SCMY/130118, cK/CH/SCYB/130321, cK/CH/SCMY/140120, cK/CH/SCLZ/121120, cK/CH/SCEM/120312, and cK/CH/SCYA/121223) were included in the QX group, sharing 94.0–99.9% nucleotide identity with the S1 sequences of other QX-like IBVs from pandemics in recent years. Four isolates (cK/CH/SCMS/130101, cK/CH/SCYB/141102, cK/CH/SCMS/150527, and cK/CH/YNKM/160426)

belonged to the TW type. Among these, cK/CH/SCYB/141102, cK/CH/SCMS/150527, and cK/CH/YNKM/160426 belonged to the TWI type, sharing 94.1–98.9% nucleotide identity with those of other TWI-type reference strains; cK/CH/SCMS/130101 grouped with TWII-type sequences, sharing 98.8–99.1% nucleotide identity with TWII reference strains. Two field isolates (cK/CH/GZLSY/160106 and cK/CH/SCDY/160426) clustered with Mass-type reference strains, sharing 96.3–99.7% nucleotide identity with Mass reference strains. One isolate (CK/CH/SCLS/140104) clustered with the J2 group, exhibiting 99.7–99.9% nucleotide identity with J2 reference strains. The phylogenetic tree is shown in Fig. 1.

3.3. S1 gene recombination analysis

Recombination events in the S1 gene were identified using the RDP 4.72 and SimPlot3.5.1 software. Simplot results were similar with RDP (Date not shown). Among the 24 field isolates, a total of eight recombinant strains were found (Fig. 2) and were mainly clustered into four groups, termed Variant-1, Variant-2, Variant-3, and Variant-4 (Fig. 1) in the phylogenetic tree. The Variant-1 group contained two isolates (cK/CH/SCMY/121225 and cK/CH/SCBZ/130423), Variant-2 contained four isolates (cK/CH/SCYB/140913, cK/CH/CQKX/150203, cK/CH/GZXF/151223, and cK/CH/SCEM/160318), and the Variant-3 and Variant-4 group contained only one isolate each cK/CH/SCTQ/120718 and cK/CH/GZLXM/160106, respectively.

In the Variant-1 group, the nucleotide sequences located at nucleotide positions 1–915 of strains cK/CH/SCMY/121225 and cK/CH/SCBZ/130423 showed high identity to the sequences of the QX strains ck/CH/LHLJ/110949 (98.0%) and ck/CH/LHB/100908 (99.6%), respectively. The nucleotide sequences located at 916–1620 nt of both strains, however, exhibited 99.0% identity with that of TW2296/95 (TWII type). The breakpoint site in both strains was located at 915 nt in the S1 subunit gene.

In the Variant-2 group, nucleotide sequences located at 1–716 nt of the four isolates cK/CH/SCYB/140913, cK/CH/CQKX/150203, cK/CH/GZXF/151223, and cK/CH/SCEM/160318 exhibited 99.2–99.6% identity with QX-type strain LC2, while nucleotide sequences located at 717–1620 nt of the four isolates shared 98.3–98.6% identity with those of cK/CH/LSC/99I-type strain SAIBK. The breakpoint site sequence in these four IBVs was located at 760 nt in the S1 subunit gene.

Table 1
Information for the 24 infectious bronchitis virus (IBV) isolates in this study.

IBV strains	Isolates date	Production type	Isolated location	Symptoms phenotype	Co-infection	Vaccine	Accession number
cK/CH/SCEM/120312	2012	Broiler	Sichuan (E'mei)	Respiratory	Single	H120	KU364606
cK/CH/SCLZ/121120	2012	Broiler	Sichuan (Luzhou)	Respiratory	MDV	H120	KU364608
cK/CH/SCMY/121225	2012	Broiler	Sichuan (Mianyang)	Respiratory; nephritis	NDV	4/91	KU364612
cK/CH/SCTQ/120718	2012	Broiler	Sichuan (Tianquan)	Respiratory	Single	H120	KU364615
cK/CH/SCYA/121223	2012	Broiler	Sichuan (Ya'an)	Respiratory	Single	4/91	KU364616
cK/CH/SCBZ/130423	2013	Broiler	Sichuan (Bazhong)	Respiratory	Bacteria	4/91	KU364604
cK/CH/SCMS/130101	2013	Broiler	Sichuan (Meishan)	Respiratory	Bacteria	H120	KU364609
cK/CH/SCMY/130118	2013	Broiler	Sichuan (Mianyang)	Respiratory	Bacteria	H120 and 4/91	KU364613
cK/CH/SCYB/130321	2013	Broiler	Sichuan (Yibin)	Respiratory	AIV (H9)	H120	KU364617
cK/CH/SCMS/140618	2014	Broiler	Sichuan (Meishan)	Respiratory	Single	4/91	KU364610
cK/CH/SCYB/140913	2014	Broiler	Sichuan (Yibin)	Respiratory	Single	H120	KU364618
cK/CH/SCDY/141030	2014	Broiler	Sichuan (Deyang)	Respiratory	Bacteria	4/91	KU364605
cK/CH/SCYB/141102	2014	Layer	Sichuan (Yibin)	Respiratory	Bacteria	H120	KU364619
cK/CH/SCLS/140104	2015	Broiler	Sichuan (Leshan)	Respiratory	AIV (H9)	H120	KU364607
cK/CH/SCMY/140120	2015	Layer	Sichuan (Mianyang)	Respiratory	Single	4/91	KU364614
cK/CH/GZGY/150203	2015	Broiler	Guizhou (Guiyang)	Respiratory	Single	H120	KU364603
cK/CH/CQKX/150203	2015	Layer	Chongqing (Kaixian)	Respiratory	Single	H120	KU364620
cK/CH/SCMS/150527	2015	Broiler	Sichuan (Meishan)	Respiratory; nephritis	Single	H120	KU364611
cK/CH/GZXF/151223	2015	Broiler	Guizhou (Xifeng)	Respiratory	Coccidiosis	H120; 4/91	KU516832
cK/CH/GZLSY/160116	2016	Broiler	Guizhou	Respiratory	Single	H120; 4/91	KX129907
cK/CH/GZLXM/160116	2016	Broiler	Guizhou	Respiratory	/	H120; 4/91	KX129908
cK/CH/SCEM/160318	2016	Broiler	Sichuan (E'mei)	Respiratory	Single	H120; 4/91	KX129909
cK/CH/YNKM/160426	2016	Broiler	Yun-nan (Kunming)	Nephritis	Single	H120; 4/91	KX344067
cK/CH/SCDY/160426	2016	Broiler	Sichuan (Deyang)	Proventriculus	Bacteria	H120; 4/91	KX344068
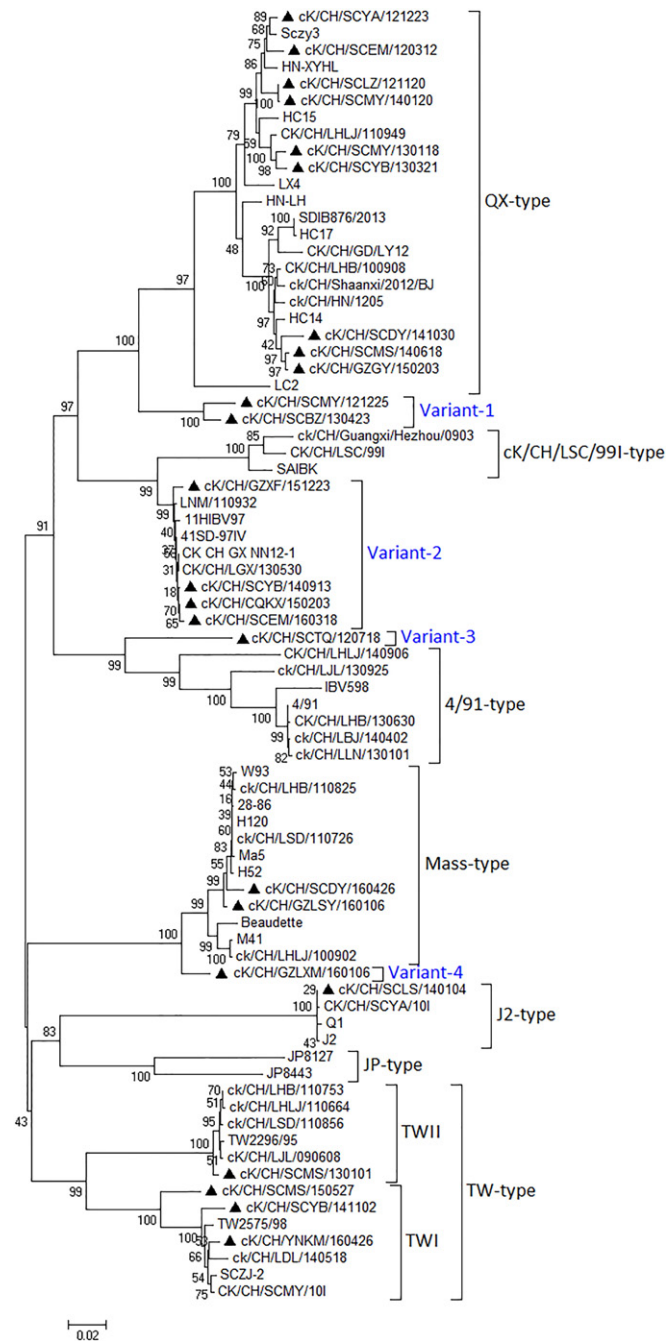
Fig. 1. Phylogenetic analysis of the S1 gene from 24 wild strains (filled triangles) and 53 reference strains of infectious bronchitis virus (IBV), starting at the AUG translation initiation codon and ending at the cleavage recognition motifs. The phylogenetic tree was constructed using MEGA version 7.0.14 with the neighbor-joining method and 1000 bootstrap replicates.

For the Variant-3 strain cK/CH/SCTQ/120718, positions 1–738 nt exhibited 97.7% identity with those of CK/CH/LSC/99I-type strain CK/CH/Guangxi/Hezhou/0903, while positions 739–1626 nt exhibited 95.0% identity with that of 4/91-type strain ck/CH/LJL/130925. The breakpoint site was at position 703 nt in the S1 subunit gene.

Finally, for the Variant-4 strain cK/CH/GZLXM/160116, positions 1–1402 nt exhibited 99.4% identity with sequences from the vaccine strain H120, while positions 1403–1611 exhibited 98.1% identity with sequences from the QX-type strain CK/CH/GD/LY12. The breakpoint site was located at 1396 nt in the S1 subunit gene.

Further analysis the sequences near the breakpoint site, revealed a new potential, A-T rich hotspot sequence, ATTTT(T/A), which was

found near the breakpoint site of the S1 subunit gene in all of the Variant-1, Variant-2, Variant-3 and Variant-4 groups (Fig. 3).

3.4. Viral cross-neutralization tests

The antigenicities of representative isolates from different genotypes were assessed by viral cross-neutralization assays (Tables 2 and 3). Ten IBVs, including seven field strains from different genotype backgrounds and three commonly used vaccine strains (H120, 4/91, and M41), were analyzed and grouped into four serotypes: Massachusetts (Mass hereafter), 793B, Sczy3, and SCYB. The commonly used vaccines H120 and 4/91 had no cross-neutralization titers and were used as reference serotypes. Four IBVs – including the vaccine strain H120, field isolates cK/CH/SCDY/141030 (QX) and cK/CH/SCLS/140104 (J2), and the standard virulent strain M41, commonly used in inactivated vaccine production – were grouped into the Mass serotype. Seven IBVs [4/91, cK/CH/SCMS/130101 (TW), cK/CH/CQKX/150203 (Variant-2), cK/CH/SCYB/140913 (Variant-2), cK/CH/SCLS/140104 (J2), cK/CH/SCYB/141102 (TW), and M41] were grouped into the 793B serotype. Similarly, seven IBVs [Sczy3, cK/CH/CQKX/150203 (Variant-2), cK/CH/SCDY/141030 (QX), cK/CH/SCLS/140104 (J2), cK/CH/SCMS/130101 (TW), cK/CH/SCYB/141102 (TW), and M41] were grouped into the Sczy3 serotype, while only three IBVs [cK/CH/SCYB/140913 (Variant-2), 4/91, and cK/CH/SCYB/141102 (TW)] were grouped into the SCYB serotype (Table 4).

Many IBVs, including six field strains and one vaccine strain (4/91) were included in different serotypes: cK/CH/SCDY/141030 (QX) was included in both the Mass and Sczy3 serotypes, cK/CH/SCLS/140104 and M41 were included in all serotypes except SCYB, cK/CH/SCMS/130101 and cK/CH/CQKX/150203 (Variant-2) were included in both 793B and Sczy3 serotypes, cK/CH/SCYB/141102 was included in all serotypes except Mass, cK/CH/SCYB/140913 and 4/91 were included in both 793B and SCYB serotypes. Only H120 and the Sczy3 standard were included in a single serotype (Table 4).

In the unidirectional neutralization assay of vaccine-associated strains, immune sera against H120 could only neutralize three IBVs from three different genotypes, while immune sera against 4/91 were able to neutralize six IBVs from five different genotypes. Although H120 and M41 belong to the same serotype, immune sera against M41 neutralized IBVs from six different genotypes, and the neutralizing titers were higher than those of H120 in most cases. The neutralization abilities of antisera against 4/91 and M41 were higher than that against H120 (Table 2).

In terms of the unidirectional neutralization assays of the seven field strains, immune sera of cK/CH/SCDY/141030, cK/CH/SCLS/140104, and cK/CH/CQKX/150203 neutralized all ten of the analyzed strains. Immune sera of cK/CH/SCYB/140913 and cK/CH/SCYB/140913 neutralized all of the analyzed strains except H120. Immune sera of cK/CH/SCMS/130101 and cK/CH/SCYB/141102 could neutralize all strains with the exception of one field strain. The neutralization abilities of antisera against most of the field strains were almost identical to that of 4/91, which was higher than that of vaccine strain H120 (Table 2).

For cross-neutralization assay, most analyzed IBVs displayed bidirectional neutralization activity, but several IBVs displayed unidirectional neutralization activity. For example, immune sera against cK/CH/CQKX/150203, cK/CH/SCMY/130101, and cK/CH/SCYB/141102 neutralized H120 well, but immune sera against H120 did not react well with these three field strains (Table 2).

Although most strains belonging to the same genotype also belonged to the same serotype (e.g. H120 and M41, Sczy3 and cK/CH/SCDY/141030) some IBVs in the same genotype exhibited low antigenic relatedness values, such as cK/CH/SCYB/140913 and cK/CH/CQKX/150203 from the Variant-2 group. In contrast, IBVs from different genotype groups sometimes also exhibited high antigenic relatedness values (r ≥ 100%), such as H120 (Mass) and cK/CH/SCLS/140104 (J2); 4/91 (4/
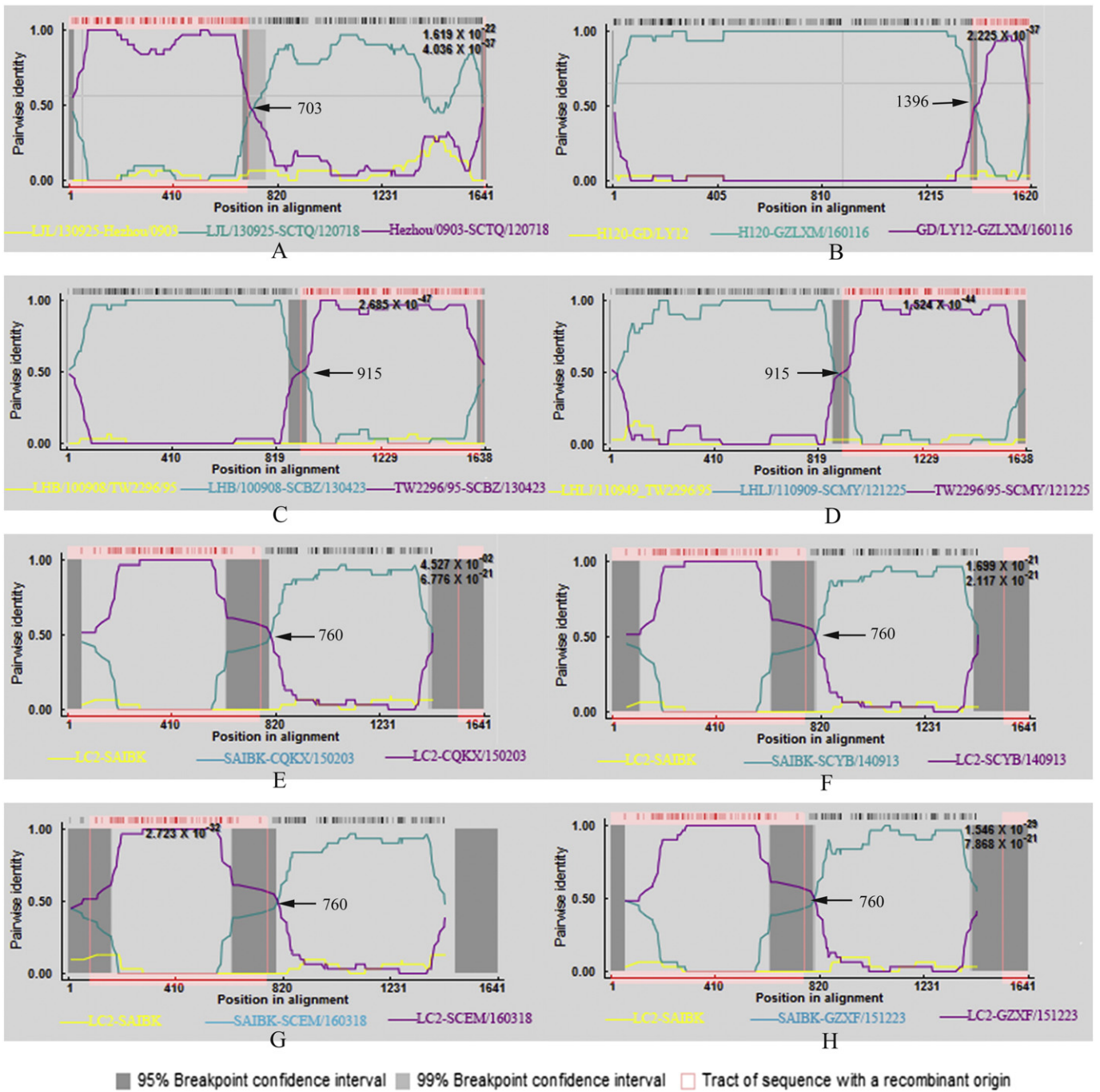
Fig. 2. RDP query displaying possible recombination events in strains (A) cK/CH/SCTQ/120718, (B) cK/CH/GZLXM/160116, (C) cK/CH/SCBZ/130423, (D) cK/CH/SCMY/121225, (E) cK/CH/CQKX/150203, (F) cK/CH/SCYB/140913, (G) cK/CH/SCEM/160318, and (H) cK/CH/GZXF/151223. The identified recombination breakpoints are shown as black arrows, highly similarity (>0.95) regions with putative parental fragments was show in dark gray area (▪), and pink box (▢) indicated the recombinant origin with putative parental strains.

91) and cK/CH/CQKX/150203 (Variant-2); and 4/91 (4/91) and cK/CH/SCMS/130101 (TW) (Table 4).

4. Discussion

In recent years, outbreaks of IB have been reported frequently in southwestern China (Zhang et al., 2016; Zou et al., 2010). Although the mortality rate of a single infection has been low, it could increase as a consequence of secondary infections or co-infection with *E. coli*, AIV, and NDV (Dwars et al., 2009; Hassan et al., 2016). In our study, rates of co-infection of IBV and bacteria accounted for 25.0% of total cases. Control of co-infections or secondary infections with other

pathogens are very important for the prevention and control of IB (Sid et al., 2015; Smith et al., 1985).

The molecular characteristics of IBVs vary around the world. Over the past ten years, epidemics of different genotypes have been observed in different countries or areas, such as the Variant-2 (IS/1494) and Mass types in Asia (Chen et al., 2015; Patel et al., 2015; Promkuntod et al., 2015; Seger et al., 2016; Xu et al., 2016b; Zou et al., 2010); the Mass and 793B types in the Middle East (Ganapathy et al., 2015; Hosseini et al., 2015; Najafi et al., 2016); the Mass, QX, and Italy-2 types in Africa (Fellahi et al., 2015a; Fellahi et al., 2015b; Knoetze et al., 2014); the 4/91, QX, and Italy-2 types in Europe (Kiss et al., 2015; Krapez et al., 2011); and the Mass type in South America (Balestrin et al., 2014) and
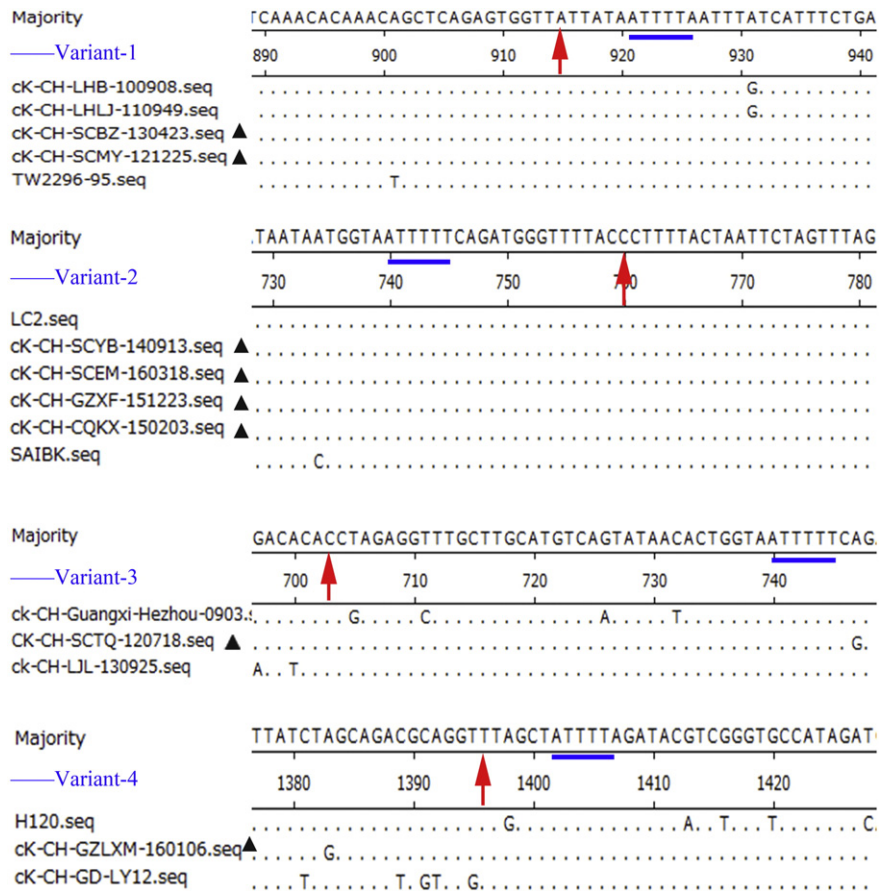
Fig. 3. Nucleotide sequence alignment of breakpoint sites of the four putative recombinant strains (solid black triangles ▲). The sequences were aligned using the Clustal W method in the MegAlign package. The hotspot sequence ATTTT(T/A) (blue underline) was found near the breakpoint site (red arrows) of the S1 subunit gene in all of the Variant-1, Variant-2, Variant-3 and Variant-4 groups. Dots represent identities with the consensus nucleotides.

North America (Mondal et al., 2013). The major genotype circulating around the world, however, is the QX type. In our study, 24 IBVs were isolated from H120- and 4/91-vaccinated chickens. Phylogenetic analysis of the S1 gene showed that those 24 IBVs could be primarily grouped into four genotypes, with QX-type strains (9/22) dominating. Many scholars have recently prepared QX vaccine strains for use as a candidate vaccine in China (Huo et al., 2016; Zhao et al., 2015), but there is still no official approval for field application of a QX-like vaccine in China. Considering the possibility of recombination between vaccine strains or between vaccine strain and field strain to generate variants, field application of new attenuated live vaccine such as QX-like vaccine should be carefully assessed before implementation.

In contrast to QX-type epidemics, epidemics of the TW type (TWI and TWII) and Variant-2 type have been increasing in recent years (Mo et al., 2013; Xu et al., 2016a), a fact that was also confirmed by this study. Most scholars classify Variant-2 strains into the cK/CH/LSC/99Itype or SAIBK type, but our RDP and Simplot analysis showed that Variant-2 strains are the result of a recombination event between a QX-type and a cK/CH/LSC/99I-type (SAIBK-type) strain. Both the TWI and TWII type were first identified in Taiwan, and the isolation of TWI-type strains in China has increased in recent years (Fu-yan, 2013; Zhang et al., 2016), while TWII-type strains were first identified in China in 2012 (Ma et al., 2012). In our study, we not only identified TWII-type strains among our samples, but we also discovered recombinant IBVs originating from QX-type and TWII-type strains. How TWII and associated recombinant strains appeared in flocks from southwestern China remains unknown. Measures should be taken to prevent the evolution of new variants and recombinant variants, such as strengthening supervision of the poultry trade, limiting the number and type

of live vaccine strains, and increasing the biosecurity level of chicken farms.

The S1 glycoprotein carries most of the neutralizing epitopes in the IBV genome, and the S1 subunit gene is highly variable. Gene mutations may be introduced into the viral genome by viral RNA-dependent RNA polymerases, which display incomplete proofreading capabilities (Denison et al., 2011(Denison et al., 2011), and gene recombination can occur via a genomic template switching mechanism (Lai, 1992). The homology sequences between the donor and acceptor genome are usually required for the copy-choice of homologous recombination, and the RNA secondary structure such as hairpin structure could also influence the recombination process (Lai, 1992; Nagy et al., 1998). Mutation and recombination of the S1 gene may lead to the emergence of new variants, genotypes, or serotypes (Zhang et al., 2015). In this study, we found that mutations in the S1 subunit gene were mainly located in the three hyper-variable regions (HVR) (data not shown), which is consistent with our previous study (Zou et al., 2010). For the recombination occurred in the S1 gene, previous studies have demonstrated that most crossover occur at a relatively conserved sequence near the HVR (Wang et al., 1994). However, this was not the case for three of the four putative recombination events detected in this study. The breakpoint site in the Variant-1 group was located at a conserved sequence in the HVR, while that in the Variant-3 group was located in a variable region outside of the HVR. Recombination hotspots are generally believed to be located adjacent to putative breakpoint sites, CT(T/ G)AACAA, CTTTTG, and CTTTTG(C/T) are usually considered to be potential hotspot sequences. In this study, the hotspot sequences above were not observed near the breakpoint sites, but a potentially new, A-T rich hotspot sequences, ATTTT(T/A), was observed near the

Table 2
The cross VN end point titers of infectious bronchitis virus (IBV).

Virus	Antisera against different IBV strains									
	Sczy3	K/CH/SCDY/141030	H120	M41	4/91	K/CH/SCLS/140104	K/CH/SCYB/140913	K/CH/CQKX/150203	K/CH/SCMS/130101	K/CH/SCYB/141102
1. Sczy3 (QX)	**168**	168	0	146	57	96	68	127	127	223
2. cK/CH/SCDY/141030 (QX)	68	**146**	96	127	47	323	42	224	146	224
3. H120 (Mass)	0	126	**146**	168	0	168	0	322	146	146
4. M41 (Mass)	168	47	224	**323**	146	224	224	160	57	57
5. 4/91 (4/91)	116	126	0	284	**224**	323	138	284	126	224
6. cK/CH/SCLS/140104 (J2)	68	96	57	168	57	**146**	57	57	126	68
7. cK/CH/SCYB/140913 (Variant-2)	80	41	57	96	395	64	**366**	32	0	96
8. cK/CH/CQKX/150203 (Variant-2)	127	43	0	57	57	57	74	**47**	57	0
9. cK/CH/SCMS/130101 (TW)	57	32	0	47	127	146	68	32	**57**	68
10. cK/CH/SCYB/141102 (TW)	96	127	0	47	68	168	146	127	68	**146**

(): Genotype of the IBV strain. Bold data means the VN end point titers of antisera against the homologous strain.

Table 3
Antigenic relatedness values (r) of ten infectious bronchitis virus (IBV) strains.

Virus	Sczy3	K/CH/SCDY/141030	H120	M41	4/91	K/CH/SCLS/140104	K/CH/SCYB/140913	K/CH/CQKX/150203	K/CH/SCMS/130101	K/CH/SCYB/141102
1. Sczy3 (QX)	100	68	0	67	42	52	30	143	87	94
2. cK/CH/SCDY/141030 (QX)		100	75	36	43	120	18	118	75	115
3.H120 (Mass)			100	89	0	67	0	0	0	0
4. M41 (Mass)				100	76	89	43	77	91	24
5.4/91 (4/91)					100	75	82	124	112	68
6. cK/CH/SCLS/140104 (J2)						100	26	69	148	73
7. cK/CH/SCYB/140913 (Variant-2)							100	37	0	51
8. cK/CH/CQKX/150203 (Variant-2)								100	82	0
9. cK/CH/SCMS/130101 (TW)									100	74
10. cK/CH/SCYB/141102 (TW)										100

(): Genotype of the IBV strain.
Strains with r ≥ 50% were classified as the same serotype, and strains with r < 50% were classified as different serotypes. Underlined data means the antigenic relatedness values of the homologous IBV strain.

Table 4
Classification of infectious bronchitis virus (IBV) serotypes.

Serotype	The classification of IBVs		
	r ≥ 100%	50% ≤ r < 100%	r < 50%
Massachusetts	H120 (Mass)	cK/CH/SCDY/141030 (QX) cK/CH/SCLS/140104 (J2) M41 (Mass)	Sczy3 (QX) 4/91 (4/91) cK/CH/SCYB/140913 (Variant-2) cK/CH/CQKX/150203 (Variant-2) cK/CH/SCMS/130101 (TW) cK/CH/SCYB/141102 (TW)
793B	4/91 (4/91) cK/CH/SCMS/130101 (TW) cK/CH/CQKX/150203 (Variant-2)	cK/CH/SCYB/140913 (Variant-2) cK/CH/SCLS/140104 (J2) cK/CH/SCYB/141102 (TW) M41 (Mass) cK/CH/SCDY/141030 (QX)	Sczy3 (QX) cK/CH/SCDY/141030 (QX) H120 (Mass)
Sczy3	Sczy3 (QX) cK/CH/CQKX/150203 (Variant-2)	cK/CH/SCLS/140104 (J2) cK/CH/SCMS/130101 (TW) cK/CH/SCYB/141102 (TW) M41 (Mass)	H120 (Mass) 4/91 (4/91) cK/CH/SCYB/140913 (Variant-2)
SCYB	cK/CH/SCYB/140913 (Variant-2)	4/91 (4/91) cK/CH/SCYB/141102 (TW)	Sczy3 (QX) cK/CH/SCDY/141030 (QX) H120 (Mass) M41 (Mass) cK/CH/SCLS/140104 (J2) cK/CH/CQKX/150203 (Variant-2) cK/CH/SCMS/130101 (TW)

( ): Genotype of the IBV strain.
Strains with r ≥ 50% were classified as the same serotype, and strains with r < 50% were classified as different serotypes.

breakpoint sites of the S1 subunit gene. Sequence analysis of recombinant IBVs in other reports (Mo et al., 2013; Thor et al., 2011) showed that ATTTT(T/A) was also observed near the breakpoint sites in some of the strains (Date not shown). Previous report had shown that AU-rich sequence could be the important recombination-promoting signals of Brome Mosaic Virus (BMV) (Shapka and Nagy, 2004).

To determine the antigenic relatedness between field strains and vaccine strains in southwestern China, viral cross-neutralization tests were performed. As most IBV isolates do not produce significant CPEs in CEK cells, IBVs should be allowed to adapt to CEK cells before neutralization assays. In this study, H120, 4/91, and M41 were used as vaccine controls, as they represent the most commonly used vaccine strains. Results of the cross-neutralization assay showed that all of the analyzed strains could be grouped into four serotypes: Mass, 793B, Sczy3, and SCYB. The cross-neutralization ability of 4/91 was higher than that of H120, which may explain why the immunogenicity of 4/91 is higher than that of H120 in field applications. However, the antigenic relatedness between some field strains and 4/91 was also low, which may explain immune failure in flocks vaccinated with 4/91. Sczy3 and SCYB were two serotypes that differed from that of the H120 and 4/91 vaccine, and the cross-neutralization abilities of strains in the Sczy3 serotype were higher than those of strains in the SCYB serotype. Representative strains in the Sczy3 serotype may therefore be more suitable for vaccine development than those in the SCYB serotype.

In general, there is a correlation between S1 gene homology and the level of cross-protection between strains, with strains in the same serotype usually sharing >95% amino acid identity and strains from different serotypes sharing <85% amino acid identity (Cavanagh, 2005). However, in this study, some strains with high amino acid identity (99.4%), such as cK/CH/SCYB/140913 and cK/CH/CQKX/150203, exhibited low antigenic relatedness, corroborating a previous study that showed that strains with low amino acid identity could also display high antigenic relatedness (Sjaak de Wit et al., 2011). It is unclear which amino acids play key roles in determining the serotype of IBVs, and genetic analysis could not be used to evaluate antigenic differences between IBVs. Furthermore, we found that some IBV strains, such as cK/CH/SCDY/141030, could be grouped into more than one serotype, similar to the results of other reports (Cowen and Hitchner, 1975; Winterfield and Fadly, 1972). This phenomenon may be explained by the existence of common VN epitopes present in different IBV genes. For example, in

cK/CH/SCDY/141030 and Sczy3, two common VN epitopes (87PPQGMAW93 and 412IQTRTEP418) (Zou et al., 2015) were observed in the S1 genes of both strains. Although the five VN epitopes in the HVRs were quite different between strains cK/CH/SCDY/141030 and H120, other common VN epitopes may be located in the S2 and N proteins of these two strains, as there are reports that the S2 and N proteins may induce neutralizing reactions (Ignjatovic and Sapats, 2005; Koch et al., 1990).

In conclusion, we have demonstrated that the genetic and antigenic characteristics of IBVs isolated from southwestern China have undergone some changes in recent years. Our results provide a reference for the prevention and control of IB in southwestern China.

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