Isolation and genetic analysis revealed no predominant new strains of avian infectious bronchitis virus circulating in South China during 2004–2008

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A R T I C L E   I N F O

A B S T R A C T

Twenty-seven strains of avian infectious bronchitis virus (IBV) were isolated from dead or diseased chickens at different chicken farms in South China during 2004–2008, of which the S1 gene was sequenced. Phylogenetic analysis of the S1 gene sequences of the isolated 27 strains together with 29 strains published in Genbank revealed that all IBV strains except for one isolated and one published were clustered into six distinct genotypes I-VI. 26 isolated strains belong to genotypes I, II, and III, forming a big phylogenetic branch without new predominant strains, whereas all five vaccine strains belong to genotype V that is evolutionarily distant from genotypes I, II, and III. The study of the protease cleavage motif within the S1 protein found 12 different cleavage motifs, of which 3 motifs are shared by both isolated and published strains, 2 motifs unique to isolated strains, and 7 motifs unique to published strains, further bolstering the notion of no new predominant strains. Alignment analysis of the S1 amino acid sequences indicated that the amino acid substitutions, insertions, and deletions are polymorphic and diverse, showing no sign of predominant genetic changes among the isolated strains. Taken together, there was no predominant new strain circulating in South China during 2004–2008. Nonetheless, circulating IBV strains have been continuously evolving with genetic compositions distant from vaccine strains; this explains why there have been constant but infrequent outbreaks in commercial flocks in South China during 2004–2008. Furthermore, in order to safe guard against the sudden emergence of new predominant strains, continuing surveillance of IBV strains circulating in the field is of extreme importance.

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1. Introduction

Avian infectious bronchitis virus (IBV), a member of the Coronaviridae (order Nidovirales, genus Coronavirus) family, is a highly infectious and contagious pathogen of domestic fowl, replicating primarily in the respiratory tract but also in some epithelial cells of the gut, kidney, and oviduct (Cavanagh, 2001; Cavanagh and Naqi, 2003; Cook et al., 2001). IBV genome consists of a single-stranded sense RNA encoding four structural proteins, envelope (E) glycoprotein, integral membrane (M) glycoprotein, phosphorylated nucleocapsid (N) protein, and spike (S) glycoprotein (Lai and Cavanagh, 1997; Stern and Sefton, 1982). S glycoprotein is posttranslationally cleaved into amino-terminal S1 (535 amino acids; 90-kDa) and carboxyl-terminal S2 (627 amino acids; 84-kDa) subunits

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by cellular proteases (Cavanagh et al., 1986b); S1 forms a receptor-binding site whereas S2 anchors S1 to the viral membrane. The protease cleavage site is usually associated with one or more pairs of basic amino acids (e.g., Arg-Arg-Ser-Arg-Arg) (Cavanagh et al., 1992).

S1 protein involves in infectivity, contains virus-neutralizing epitopes, serotype-specific sequences, and hemagglutinin activity (Cavanagh, 1983; Cavanagh and Davis, 1986; Cavanagh et al., 1986a; Karaca et al., 1992; Koch et al., 1990), and plays a major role in the tissue tropism and the induction of protective immunity (Cavanagh et al., 1988, 1992). As the most variable protein, S1 protein sequences from different strains vary significantly, usually by between 2 and 25% at the amino acid level (Lai and Cavanagh, 1997). Various serotypes are believed to be generated by nucleotide insertions, deletions, point mutations, and/or RNA recombinations in S1 subunit (Jia et al., 1995; Kusters et al., 1987; Wang et al., 1993, 1994). The mutations in the N-terminal amino acids of S1 subunit directly lead to emergence of new serotypes and change of tissue tropism (Casais et al., 2003; Cavanagh et al., 1986a; Stern and Sefton, 1982). Certain studies indicated that IBV immunity was serotype-specific, small differences in S1 contributing to poor cross-protection (Cavanagh and Davis, 1986; Cavanagh et al., 1997; Gelb et al., 1997; Wang et al., 1994) and vaccine failures (Jia et al., 1996), while some groups reported that cross-protection could be provided by appropriate vaccine programmes against genetically or antigenically unrelated IBVs (Gelb et al., 2005; Cook et al., 1999). S1 gene sequence was reported as a good predictor of challenge of immunity in chickens (Ladman et al., 2006), the genetic analyses of IBV strains have been mainly focused on the S1 gene (Cavanagh et al., 1986b, 1997; McFarlane and Verma, 2008).

Since IBV strains were first isolated and identified in China in 1982, infectious bronchitis (IB) caused by IBV has been an economically important disease to the poultry industry. Vaccines based on Massachusetts (Mass) serotype strains such as H120 and H52 have been used for many years worldwide (Hofstad, 1981). Moreover, various live or inactivated vaccines based on Mass serotype strains have been used for control of IBV infections in China. Vaccines have been generally effective, but new strains continue to emerge and cause clinical disease and production problems in vaccinated flocks (Bijlenga et al., 2004; Gelb et al., 2005; Nix et al., 2000). For example, based on neutralization test and the immune protection test, A2 strain is closely related to 4/91 serotype, spreading over Europe since its first isolation in UK in 1991 (Capua et al., 1999; Cavanagh et al., 2005; Cook et al., 1996; Gough et al., 1992; Liu et al., 2009b; Meulemans et al., 2001; Parsons et al., 1992; Xu et al., 2007; Zhao and Qin, 2002). QXIBV, a new IBV variant reported in China in 2004 (Liu and Kong, 2004), had become widespread in several countries in Europe (Terregino et al., 2008; Worthington et al., 2008). In recent years, nephropathogenic IBV strains had been circulating in China (Bing et al., 2007; Liu and Kong, 2004; Liu et al., 2006). However, the natures of circulating IBV strains in South China were not clear.

This study took on the task of deciphering the natures of the IBV strains circulating in commercial flocks in South China by carrying out a long-term surveillance program. During the program, 27 IBV strains were isolated in South China from clinical outbreaks occurred in the period of 2004–2008. The isolated IBV strains were molecularly characterized by sequencing the whole S1 genes. Sequence alignment and phylogenetic analysis of the isolated IBV strains together with other published IBV strains provided useful information as to the nature of the circulating IBV strains; and the implications of this study in the strategies for future protection of IBV infections are also discussed.

2. Materials and methods

2.1. Virus

Circulating field IBV strains were isolated from dead or diseased broilers at different chicken farms located in Guangdong, Guangxi, Sichuan, Hainan, Chongqing, and Hubei provinces during 2004–2008 (Table 1). Documented clinical signs included typical respiratory and nephropathogenic IB symptoms and pathological changes. Viruses were propagated by more than three passages in the allantoic cavities of 10-day-old specific pathogen free (SPF) chicken embryos. The allantoic fluid was collected after 48 h post-inoculation, frozen at $-80\,°C$ for RNA extraction, and viruses were identified as IBV by the observation of curled and dwarfed embryos and RT-PCR for the N protein gene.

2.2. Primers for S1 gene

A pair of primers for amplifying entire S1 gene were 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 the entire S1 gene including the protease cleavage motif.

2.3. RNA extraction, RT-PCR and PCR

Viral RNA was extracted using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The first-strand cDNA was synthesized using an PrimeScript[TM] 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). According to the manufacturer's instructions, reverse transcription reaction mixture was set up as follows: 1 μl of Random 6 mers (50 μM), 1 μl of 10 mmol/L dNTPmix, 2 μl of RNA extract, 6 μl RNase free dH$_2$O. The reaction mixture was incubated at 65 °C for 5 min, and then quickly frozen on ice. After cooling, reagents were added in the same tube as follows: 4 μl 5× PrimeScript Buffer, 0.5 μl of 40 U/μl RNase inhibitor, 1 μl of 200 U/μl PrimeScript Rtase, 4.5 μl RNase free dH$_2$O. The total volume of reverse transcription reaction mixture was 20 μl; the reaction was carried out at 30 °C for 10 min, 42 °C for 30 min, 70 °C for 15 min. About 1 μl of the cDNA was used for amplification in PCR reactions. The PCR reactions (total volume of 50 μl) contained 5 μl of 10× PCR buffer,

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

IBV isolates	Province[a]	Years of isolation	Major clinical signs	Spike glycoprotein cleavage recognition motifs	Accession number
CQ04-1	Chongqing	2004	Nephritis	RRFRR[1]	GQ265952
CQ04-2	Chongqing	2004	Nephritis	RRSKR[3]	GQ265953
DY04	Sichuan	2004	Nephritis	RRFRR[1]	GQ265950
NN04	Guangxi	2004	Nephritis	RRSRR[4]	GQ265951
DY05	Sichuan	2005	Nephritis	HRRRR[2]	GQ265928
GM05	Guangdong	2005	Nephritis	RRFRR[1]	GQ265931
LZ05	Guangdong	2005	Nephritis	RRFRR[1]	GQ265943
HN06-1	Hainan	2006	Nephritis	RRFRR[1]	GQ265938
HN06-2	Hainan	2006	Nephritis	RRFRR[1]	GQ265939
HY06	Guangdong	2006	Nephritis	RRSKR[3]	GQ265941
SS06-1	Guangdong	2006	Respiratory	RRSKR[3]	GQ265935
SS06-2	Guangdong	2006	Respiratory	RRFRR[1]	GQ265937
DY07	Sichuan	2007	Respiratory	HRRRR[2]	GQ265927
HY07	Guangdong	2007	Nephritis	RRSKR[3]	GQ265942
LZ07	Guangdon	2007	Respiratory	RRFRR[1]	GQ265944
NN07	Guangxi	2007	Nephritis	HRRRR[2]	GQ265946
SS07	Guangdong	2007	Respiratory	RRFRR[1]	GQ265936
TC07-1	Guangdong	2007	Nephritis	HRRRR[2]	GQ265947
TC07-2	Guangdong	2007	Respiratory	HRRKR[5]	GQ265948
ZX07	Guangdong	2007	Nephritis	HRRRR[2]	GQ265949
FS08-1	Guangdong	2008	Respiratory	RRFRR[1]	GQ265929
FS08-2	Guangdong	2008	Respiratory	RRFRR[1]	GQ265930
GL08-1	Guangxi	2008	Respiratory	HRRRR[2]	GQ265932
GL08-2	Guangxi	2008	Respiratory	HRRRR[2]	GQ265933
HB08	Hubei	2008	Respiratory	HRRRR[2]	GQ265934
HN08	Hainan	2008	Nephritis	RRFRR[1]	GQ265940
XX08	Guangdong	2008	Nephritis	RRFRR[1]	GQ265945

[a] Province where the viruses were isolated.
[1] RRFRR: Arg-Arg-Phe-Arg-Arg (13).
[2] HRRRR: His-Arg-Arg-Arg-Arg (8).
[3] RRSKR: Arg-Arg-Ser-Lye-Arg (4).
[4] RRSRR: Arg-Arg-Ser-Arg-Arg (1).
[5] HRRKR: His-Arg-Arg-Lys-Arg (1).

4 μl of 2.5 mmol/l dNTP, 2 μl of 10 μmol/l of each of the two primers, and 0.25 μl of 2 U/μl Taq DNA polymerase. The PCR conditions for amplification were 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 10 min. The products were analyzed on 1.0% agarose gel.

2.4. DNA cloning

PCR products of each RT-PCR were purified using Axy Prep™ DNA Gel Extraction Kit (AXYGEN). Purified PCR products ligated with a TA cloning vector, pMD18-T (TaKaRa, Japan), were transformed into DH5a *E. coli* competent cells. Cells carrying recombinant plasmid were screened on Luria-bertani (LB) agar plates containing Ampicillin (50 μg/ml). Positive clones were initially screened from three different PCRs through blue and white colonies; then the inserts were amplified by PCR which had the same conditions as that for the above-mentioned PCR amplification. Positive colonies were verified by EcoRI (TaKaRa, Japan) restriction enzyme digestion of the amplified inserts. The verified recombinant plasmid DNAs were used for sequencing.

2.5. Gene sequencing and analysis

The purified recombinant plasmids were sequenced by the dideoxy chain termination procedure using the automated ABI Prism 3730, Genetic Analyzer. Translation of the nucleotide sequence and sequence alignments was performed by the DNAStar software (DNASTAR Inc., USA).

Phylogenetic analyses were performed with the neighbor-joining method using MEGA version 4. The bootstrap values were determined from 1000 replicates of the original data.

2.6. IBV strains published in Genbank

Twenty-nine IBV strains published in Genbank were selected for the performance of phylogenetic and alignment analyses, including Beaudette, M41, Gray, Holte, Ark99, 4/91, 7/93, H120, H52, Ma5, W93, QXIBV, ZJ971, LX4, A2, SC021202, SAIBK, KQ6, CK/CH/LGD/04III, CK/CH/LGD/04II, CK/CH/LLN/06I, CK/CH/LGX/06I, SDZB0804, DE/072/92, Italy-02, K069-01, JP9578, 3468/07, and T07/02 (Table 2).

3. Results

3.1. Twenty-seven IBV strains isolated during 2004–2008 in South China

From dead or diseased chickens suspected of IBV infection, 27 circulating field IBV strains were isolated from different chicken farms located in Guangdong, Guangxi, Sichuan, Hainan, Chongqing and Hubei provinces

Table 2
IBV strains published in Genbank.

IBV strains (country of origin)	Years of isolation	Serotype/Genotype/Pathogenicity type/organs used for virus isolation	Spike glycoprotein cleavage recognition motifs	Accession number
Beaudette (USA)	1937	Mass serotype	RRFRR[1]	NC_001451
M41 (USA)	1956	Mass serotype	RRFRR[1]	DQ834384
Gray (USA)	1962	Gray serotype	RRSRR[4]	L14069
Hotle (USA)	1962	Hotle serotype	RRSRR[4]	L18988
Ark99 (USA)	1973	Arkansas serotype	HRSRR[6]	M99482
4/91 (UK)	1992	793/B serotype	RRSRR[4]	AF093794
7/93 (UK)	1993	793/B serotype	RRSRR[4]	Z83979
H120	Vaccine strain	Mass serotype	RRFRR[1]	M21970
H52	Vaccine strain	Mass serotype	RRFRR[1]	AF352315
Ma5	Vaccine strain	Mass serotype	RRFRR[1]	AY561713
W93 (China)	Vaccine strain	Nephropathogenicity	RRFRR[1]	AY427818
QXIBV (China)	1997	Proventriculus	HRRRR[2]	AF193423
ZJ971 (China)	1997	Proventriculus	RRFRR[1]	AF352313
LX4 (China)	1999	Nephropathogenicity	HRRRR[2]	AY189157
A2 (China)	1996	4/91 serotype	HRRRR[2]	AY043312
SC021202 (China)	2002	Nephropathogenicity	RRHRR[7]	AY237817
SAIBK (China)	Nd[a]	Nephropathogenicity	RRFRR[1]	DQ288927
KQ6 (China)	2003	Respiratory	RRFRR[1]	AY641576
CK/CH/LGD/04III (China)	2004	Kidney	RRLRR[8]	DQ167135
CK/CH/LGD/04II (China)	2004	Kidney	RRFRR[1]	DQ167134
CK/CH/LLN/06I (China)	2006	Trachea[a]	HRPRR[9]	EF213566
CK/CH/LGX/06I (China)	2006	Respiratory	RRFRR[1]	EF213580
SDZB0804 (China)	2008	Kidney[a]	HRRRR[2]	FJ210647
DE/072/92 (USA)	1992	DE072 serotype	RRIRR[10]	AIU77298
Italy-02 (Italy)	1999	Respiratory	HRFRR[11]	AJ457137
K069-01 (Korea)	2001	Korean Genotype III	RRFRR[1]	AY257061
JP9758 (Japan)	1995	Nephropathogenicity	RRFKR[12]	AY296746
3468/07 (Taiwan)	2007	Nd[a]	RRSRR[4]	EU822336
T07/02 (Taiwan)	2002	TW II	RRFRR[1]	AY606322

[a] Nd, not documented.
[1] RRFRR: Arg-Arg-Phe-Arg-Arg (13).
[2] HRRRR: His-Arg-Arg-Arg-Arg (4).
[4] RRSRR: Arg-Arg-Ser-Arg-Arg (5).
[6] HRSRR: His-Arg-Ser-Arg-Arg (1).
[7] RRHRR: Arg-Arg-His-Arg-Arg (1).
[8] RRLRR: Arg-Arg-Leu-Arg-Arg (1).
[9] HRPRR: His-Arg-Pro-Arg-Arg (1).
[10] RRIRR: Arg-Arg-Ile-Arg-Arg (1).
[11] HRFRR: His-Arg-Phe-Arg-Arg(1).
[12] RRFKR: Arg-Arg-Phe-Lys-Arg(1).

of South China during 2004–2008; the main features of the isolated IBV strains are summarized in Table 1. Eleven strains were from chickens showing typical respiratory clinical signs such as difficulty in breathing, nasal discharge, sneezing, tracheal rales, watery eyes, lethargy, and mucosal thickening with serous or catarrhaeal exudates in the nasal passages, sinuses, and trachea. Sixteen strains were from chickens showing typical nephritis symptoms such as enlarged and pale kidneys, frequently with urate deposits in the tubules, severe dehydration and weight loss. All 27 isolated strains were verified by the observation of curled and dwarfed embryos and RT-PCR for the N gene of IBV.

3.2. Wide range of homologies among S1 nucleotide and deduced amino acid sequences

S1 gene sequences of the 27 isolated IBV strains were delineated and submitted to the GenBank database (Table 1). Sequencing results showed that S1 genes contain mutations, insertions and/or deletions, resulting in different lengths of nucleotides and consequently different numbers of amino acids being encoded. As shown in Table 3, S1 gene of the isolated strains contain 1617, 1620, 1626, 1632, 1638 nucleotides, and 539, 540, 542, 544, 546 amino acids, respectively. The similarity of the nucleotide and deduced amino acid sequences of S1 genes (data not shown) among the 27 isolated strains were 65.4–99.9% and 57.2–99.8%, respectively, indicating low homology and high variation among these strains. When the S1 genes of the 29 published

Table 3
Different lengths of nucleotides and deduced amino acids of S1 glycoprotein gene of the 27 isolated IBV strains.

Length (nt/aa)	Strains
1617/539	DY05, LZ07, ZX07
1620/540	DY07, GM05, HB08, LZ05, NN07, SS06-2, SS07, TC07-1
1626/542	CQ04-1, CQ04-2, DY04, FS08-1, FS08-2, GL08-1, GL08-2, HN06-1, HN08, HN06-2, NN04, XX08
1632/544	HY06, HY07, SS06-1
1638/546	TC07-2

IBV strains (Table 2) were included, the similarity of the nucleotide and amino acid sequence among the 56 isolates were 60.9–99.9% and 45.4–99.8%, respectively.

3.3. 26 of the 27 isolated IBV strains grouped into three genotypes by phylogenetic analysis

Phylogenetic analysis was performed on S1 gene nucleotide sequences consisting of the 27 isolated and 29 published IBV strains. All IBV strains except for the isolated TC07-2 strain and the reference DE/072/92 strain were clustered into six distinct genetic groups or genotypes (I–VI) (Fig. 1). Genotype I was comprised of 10 isolated IBV strains and 5 published IBV strains from China, designated as A2-like group for the inclusion of A2 strain; genotype II of 2 isolated and 4 published IBV strains,

designated as 4/91 group for the inclusion of 4/91 strain; genotype III of 14 isolated and 5 published strains; genotype IV of 2 published IBV strains; genotype V of 8 published IBV strains; and genotype VI of 4 published IBV strains. There are four noteworthy features of the constructed phylogenetic tree. First, all isolated IBV strains except for TC07-2 strain belong to genotypes I, II and III, forming a large evolutionary branch. Furthermore, the isolated IBV strains grouped in genotype I were most from 2007(5/10), and most of the isolated IBV strains from 2008(6/7) were grouped into genotype III, showing that there were no predominant new strains circulating in the fields of South China during 2004–2008. Second, 2 published IBV strains from Taiwan formed a unique genotype (i.e., genotype IV), implying that the evolution process in Taiwan is an isolated one. Third, all 5 known

Fig. 1. Phylogenetic tree constructed by neighbor-joining method (Mega 4.0). S1 nucleotide sequences of the 27 isolated and 29 published IBV strains were included for the phylogenetic analysis, where the 27 isolated IBV strains are marked with ante-black triangle.

vaccine strains (*i.e.*, H52, H120, M41, Ma5 and W93) were clustered into genotype V that is evolutionarily distant from genotypes I, II and III; this may explain why the isolated IBV strains survived the vaccination by the vaccine strains. Finally, 3 classical American and 1 Japanese strains formed genotype VI, and the isolated TC07-2 and published DE/072/92 strains showed the greatest evolutionary distances to all six major genotypes, but their significances are not clear. The results strongly suggest that different genotypes of IBVs were cocirculating in chicken flocks in South China.

3.4. Polymorphic protease cleavage recognition site motifs in S proteins

The spike glycoprotein of IBV is translated as a precursor protein (S0) and then cleaved into two subunits S1 and S2 by the protease during viral maturation (Cavanagh et al., 1986b). A motif at the cleavage site of the IBV S protein was generally comprised of RRXRR/S (X for any amino acid). Cleavage site motifs of the 27 isolated and 29 published IBV strains are listed in Tables 1 and 2, respectively. The 27 isolated strains contain 5 cleavage site motifs while the 29 published strains have 10 cleavage site motifs. The cleavage site motifs have the following four noteworthy features. First, in light of a total of 12 cleavage site motifs identified, the protease cleavage site motifs in S proteins are polymorphic. Second, 3 cleavage site motifs (RRFRR, HRRRR and RRSRR) are shared by both the isolated and published strains, where the shared motifs represented the majority of the strains (22/27 for isolated strains; 22/29 for published strains), and the majority of motifs have the recognized motif sequence RRXRR/S (14/ 27 for isolated strains; 21/29 for published strains). Third, 2 new motifs (RRSKR and HRRKR) emerged in the isolated strains, and in the meanwhile 7 motifs (HRSRR, RRHRR, RRLRR, HRPRR, RRIRR, HRFRR and RRFKR) identified in the published strains were not found in the isolated strains, indicating that the cleavage site motifs were undergoing a continuous evolution process. Finally, individual strains with high similarities in the phylogenetic tree usually share the same motifs, *e.g.*, the lower branch of genotype I (RRFRR), the top branch of genotype III (RRSKR), the bottom branch of genotype III (RRFRR), and the genotype V (RRFRR). However, individual strains within one genotype have multiple motifs, *e.g.*, genotype I having three motifs (RRFRR, HRRRR, and HRPRR), genotype II having 4 motifs (RRFRR, RRSRR, RRLRR, and HRFRR), and genotype III having 4 motifs (RRFRR, HRRRR, RRSKR, and RRHRR); indicating that the contribution of the cleavage site motif to one strain's genotype is limited.

3.5. Alignment analysis of nucleotide and deduced among amino acid sequence of S1 protein

Alignment analysis of S1 complete nucleotide and deduced amino acid sequences of all 27 isolated and 29 published IBV strains were performed. Strains in genotypes I–VI showed amino acid similarities of 84.6–99.8%, 78.9–94.8%, 82.6–99.8%, 84.9%, 94.4–99.6% and 77.2–80.7%, respectively. The S1 proteins of the 27 isolated strains had amino acid sequence similarities of 46.4–99.8%, and aa similarities of 58.7–79.5% to the Mass-type vaccine strain H120. The alignment analysis of the deduced amino acid sequences revealed three high variable regions located between amino acid residues 53–76, 94–141, and 253–292 (numbering using S1 sequence of Mass 41 as reference); the featured deletions, insertions and mutations were summarized in Table 4. In line with the phylogenetic analysis discussed earlier, 27 isolated strains share no similarities in the listed amino acid positions with vaccine strains (*e.g.*, H120) in genotype V. Interestingly, TC07-2 strain had a 4 amino acids insertion (Gln-Lys-Glu-Pro) at the region of 283–284, and this insertion was reported for the first time from this study (Table 4); the significance of this insertion in vial pathogenicity is not clear yet. Moreover, BLAST searches revealed that TC07-2 did not show close similarity (<80%) to any S1 gene sequences available.

4. Discussion

This paper is a periodic report on our ongoing surveillance program with dual objectives, identifying the IBV strains that have escaped immune defenses conferred by vaccination and have caused persistent but infrequent outbreaks in commercial chicken farms, and preparing for the possible challenges of epidemic or pandemic IBV outbreaks in case of the emergence of a new predominant IBV strain in the field. Our survey included only broilers because most of chicken flocks in poultry industry in South China were broilers. The inclusion of only broilers in our survey did not mena that IBV was not associated with disease problems in laying and breeding chickens in South China; instead, we believe that our survey of broilers was indicative for all chickens including laying and breeding chickens. Our data showed that all 27 isolated IBV strains from South China during 2004–2008 were evolutionarily distant from the vaccine strains used for current vaccinations, explaining at least partially why vaccination failed in occasions where new IBV strains emerged and caused persistent but infrequent outbreaks. Our data also demonstrated that no new predominant IBV strains were being emerged during 2004–2008, but the continuing evolution of the escaped IBV strains makes the emergence of a new predominant strain as a matter of time, mandating of our efforts in carrying out continuing surveilance of emerging new IBV strains in order to prevent possible epidemic or pandemic outbreaks of IBV infections in commercial flocks.

In China, IB was first reported in 1972, and IBVs were found nearly all over the country in the following years. IBV infection is commonly followed by secondary bacterial infection, such as with Escherichia coli (Smith et al., 1985; Vandekerchove et al., 2004), resulting in complicated morbidity and increased mortality. Live-attenuated and inactivated vaccines have been widely used, resulting in effective control of IB (Cook, 2001). However, the outbreaks of IBV infection has been persistent but infrequent in vaccinated flocks in recent years (Liu and Kong, 2004; Liu et al., 2006, 2009a; Bing et al., 2007; Xu et al., 2007), causing clinical disease and production problems in vaccinated

Table 4
Sequence alignment of amino acid residues of the S1 glycoprotein of IBV strains with the H120 vaccine strain.

Strains	52–55	71–76	106–107	117–118	140	185	253	281	284–285	317	Genotype
H120	ISSE	HGG——————RVV	DT	–HV	–.	S.	Q	N	——PS	E	V
DY07	STNY	KDV…….**..**YNQ	EI	SVAG			D	Q	…**.**SG	A	I
NN07	STNY	KDV…….**..**YNQ	EI	SVAG	.	.	D	Q	…**.**SG	A	I
DY05	STNY	KDV…….**..**YNQ	EI	SGAG	.	.	D	Q	…**.**SG	A	I
ZX07	STNY	KDV…….**..**YNQ	EI	SGAG	.	.	D	Q	…**.**SG	A	I
HB08	STNY	KDV…….**..**YNQ	EI	SGTG	.	.	D	Q	…**.**SG	A	I
SDZB0804	STNY	KDV…….**..**YNQ	EI	SGTG	.	.	D	Q	…**.**SG	A	I
CK/CH/LLN/06I	STNY	KDV…….**..**YNQ	EI	SGAG	.	.	D	Q	…**..**IG	A	I
A2	STNH	KDV…….**..**YNQ	EI	SGTG	.	.	E	Q	…**..**SG	A	I
QXIBV	STNY	KDV…….**..**YNQ	EI	SGSG	.	.	E	Q	…**.**SG	A	I
LX4	S-NY	KDV…….YNQ	EI	SGSG	.	.	E	Q	…**.**SG	A	I
TC07-1	STNY	KDV…….YNQ	EI	SGRG	.	.	E	Q	…**.**SG	A	I
GM05	STNY	KDV…….YNQ	EI	IGTG	.	.	E	Q	…**.**SG	A	I
LZ05	STNY	KDV…….YNQ	EI	SGIG	.	.	E	Q	L…VG	H	I
SS07	STNY	KDV…….YNQ	EI	SGTG	.	.	E	Q	L…VG	H	I
SS06-2	STNY	KDV…….YNQ	EI	SGTG	.	.	E	Q	L…VG	H	I
LZ07	STNY	KDV…….YNQ	EI	SGTG	.	.	G	S	…**.**SG	P	II
CK/CH/LGD-04III	STNK	FNYSNGN.DVGYNN	.F	N—	.	G	D	S	…**.**SG	P	II
NN04	STNK	FNYTNGN.DVGYNN	.F	N—	.	G	D	S	…**.**SG	P	II
Italy-02	V.T.	SWS…….**..**KNF	.F	SGTG	S	G	D	P	S——G	P	II
4/91	VFNG	YES.…**..**YNI	.F	SQQG	F	G	D	S	S…**.**G	P	II
7/93	VFNE	YES.…….**..**HNI	HV	SQPG	V	G	D	S	S…**.**G	P	II
K069-01	STNY	KDV…….YNQ	EI	RGSG	.	.	.	S	…**.**SG	.	III
HY06	FTNK	FNYSNGN-DIGYNN	.F	N—	.	G	K	A	…**.**TG	Q	III
CQ04-2	FTNK	FNYSNGN-DIGYNN	.F	N—	.	G	K	A	…**.**TG	Q	III
SS06-1	FTNK	FNYSNGN-DIGYNN	.F	N—	.	G	K	A	…**.**TG	Q	III
HY07	FTNK	FNYSNGN-DIGYNN	.F	N—	.	G	K	A	…**.**TG	Q	III
SC021202	STNR	FNYTNGN-DVGYNN	.V	N—	.	G	E	S	…**.**QG	Q	III
SAIBK	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	E	S	…**.**QG	.	III
GL08-1	YTNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
GL08-2	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
XX08	STNR	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
CK/CH/LGX/06I	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
CK/CH/LGD/04II	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
DY04	STNK	FNYNNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
FS08-1	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
FS08-2	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
HN06-2	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
CQ04-1	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
HN06-1	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
HN08	STNK	FNYTNGN-DVGYNN	.F	N—	.	G	K	P	…**.**QG	Q	III
T07/02	VTTR	Q.………**.**Y.F	EV	STQG	.	.	.	S	…**.**PG	.	VI
3468/07	V..Q	S.D.…**..**T.F	.F	SGSG	.	.	.	Q	…**.**SG	.	VI
M41	…**..**	…………**..**	**..**	**..**YD	.	.	.	.	…..…	.	V
KQ6	…**..**	…………**..**	**..**	**..**YD	.	.	.	.	…..…	.	V
Beaudette	…**..**	…………**..**	**..**	…G	.	.	.	.	…..…	.	V
H52	…**..**	…………**..**	**..**	…**..**	.	.	.	.	…..…	.	V
ZJ971	…**..**	D.…………**..**	**..**	…**.**	.	.	.	.	…..…	.	V
Ma5	…**..**	…………**..**	**..**	…G	.	.	.	.	…..…	.	V
W93	…**..**	**..**S.………**..**	**..**	**..**QG	.	.	.	.	…..…	.	V
JP9758	V.Q.	.WS.…**..**KNF	NI	SGNN	D	G	E	P	T…VG	.	IV
Gray	V.E.	FWS.…**..**KNF	HF	VGAG	H	G	D	P	N…-G	A	IV
Ark99	V…	GYS.…**..**KNF	SY	SGSN	H	A	D	P	T…-G	.	IV
Holte	VFQ.	GWS.…**..**KNF	.I	HDG-	D	A	D	D	T…-G	.	IV
TC07-2	FFN.	VHS.…**..**LN.	TI	NRQG	N	G	E	Q	QKEPSP	S	
DE/072/92	…K	G.………VTI	SF	TG-H	Q	A	N	E	G…–	.	

Dot (.) indicates amino acid identical to that of H120 strain.
Dash (-) indicates amino acid deletion in comparison with H120 strain.

flocks (Nix et al., 2000; Farsang et al., 2002). The isolation of IBV strains from vaccinated flocks in China in recent years (Liu and Kong, 2004; Liu et al., 2006, 2009a; Bing et al., 2007; Xu et al., 2007) demonstrated that the evolution of IBV strains in the vaccinated flocks might eventually break down the immune defenses conferred by current vaccines, urging the investment of our efforts in tracking the IBV strains circulating in the commercial flocks.

In this study 27 IBV strains were successfully isolated from dead or diseased chickens from different chicken farms in South China during 2004–2008, of which S1 genes were sequenced. Phylogenetic analysis indicated that the 27 isolated IBV strains were concentrated in genotypes I, II and III, and had a large evolution distance to genotype V which is comprised primarily of vaccine strains (Fig. 1). Phylogenetic analysis also showed that the 27 isolated IBV

strains had diverse genetic origins for the isolated viruses were dispersed in three genotypes (genotype I being comprised of 3 strain from 2005, 5 from 2007, 1 from 2006 and 1 from 2008; genotype II of 1 strain from 2004 and 1 strain from 2007; and genotype III of 3 strains from 2004, 4 from 2006, 1 from 2007, and 6 from 2008). It is plausible to presume that if one strain becomes prodominant, more and more isolated strains shall be concentrated into one group as the time progresses. However, the phylogenetic analysis failed to show such characteristics. Therefore, it is safe to conclude that there were no predominant strains circulating in commercial chicken farms in South China during 2004–2008.

S protein has a protease cleavage site motif that is essential for cleaving it into S1 and S2 subunits during viral maturation (Cavanagh et al., 1986b). There was report on study of the relationship between the cleavage site motifs and host cell range, serotype, geographic origin, and pathogenicity (Jackwood et al., 2001). The analysis of the cleavage site motifs in the 27 isolated IBV strains revealed two new cleavage site motifs, showing continuing evolution of IBV strains in the fields. The most dominant motif (RRFRR) in the 27 isolated IBV strains is shared by the majority of published IBV strains. Interestingly, the motif (HRRRR) is only present in IBV strains isolated in China, implying the continuity of evolution; its significance in viral pathogenicity and vaccine preparation is not clear yet. In addition, the cleavage site motifs do not correlate with the genotypes for one genotype has multiple motifs. This is not a total surprise since it was reported that the cleavage site motifs had no relationship with pathogenicity and tissue tropism (Jackwood et al., 2001). Furthermore, respiratory and nephropathogenic strains shared the cleavage site motifs, indicating that the cleavage site motif was not a determinant to pathotypes.

The genetic diversity of the 27 isolated strains was manifested by the low similarities of S1 genes. S1 genes of the 27 isolated strains had average amino acid sequence similarities of 79.6% (varied from 0.2 to 54.6%), and amino acid similarities of 58.7–79.5% to the Mass-type vaccine strain H120, which has been widely used for vaccine production in China for decades, indicating that point mutations, deletions, and insertions of S1 protein were frequent in IBVs in South China under vaccination. Previous study showed that the first 300 amino acids in the N-terminal of the S1 protein of IBV had a high frequency of variations, called highly variable region (HVR) (Kusters et al., 1989). Analysis of the S1 amino acid sequences in this study indicated that the three hypervariable regions (HVRs) in S1 protein in this study were similar to previous studies (Cavanagh et al., 1988; Moore et al., 1997; Liu et al., 2006; Schikora et al., 2003).

The reisolation of vaccine virus was an important issue for our survey program. This was addressed in two ways: genetic analysis and phylogenetic tree clustering. For S1 gene, no field isolate showed more than 84% (data not shown) similarity of nucleotide sequence to vaccine strains (genotype V), and no field isolate was clustered to vaccine strains in phylogenetic tree. Therefore, we could reasonably rule out that any field isolate was a reisolation of vaccine virus. Nonetheless, the fact that NN04 and LZ07

were grouped with 4/91 in Group II in Fig. 1 was intriguing because it was possible they might be originated from the leak of 4/91 strain from experiments done in China even though no vaccine based on 4/91 strain was licensed in China. This possibility was highly unlikely because their S1 amino acid sequences were very different. As shown in Table 4, amino acids from 52 to 55 and from 71 to 76 were VFNG and YES......YNI for 4/91, STNK and FNYTNGN.DV-GYNN for NN04, and STNY and KDV.......YNQ for LZ07.

QXIBV was first isolated in China in 1996 (reported in Chinese), associated with proventriculitis. In 2004, this virus was reported associated predominantly with various forms of renal pathology in China (Liu and Kong, 2004). The QX-like IBV strains more recently have been widely detected in many European countries, becoming a dominant genotype in some European countries, and are causing severe losses to both the layer and broiler industry (Worthington et al., 2004; Worthington and Jones, 2006). In addition, QX-like strains have been reported associated with false layers, swollen kidneys. In the present study, 6 field isolates were clustered to QXIBV in a branch (Fig. 1), of which, 4 show typical nephritis symptoms and 2 show typical respiratory clinical signs. In Worthington et al. (2008), the European QX-like IBV strains have been detected in flocks of broilers, broiler breeders and commercial layers. Of these flocks, 86% had respiratory signs, 22% had wet litter or enteric problems, 14% had increased mortality, 2% had swollen kidneys and 2% had arthritis. In 60% of the layers there was a reduction of egg production and quality. In the phylogenetic tree analysis with QX-like IBV strains from both Europe and China (data not shown), it is of interest that the QX-like IBV strains disclosed in Worthington et al. (2008) were clustered into a different branch than the one formed by the isolates from China, indicating that the QX-like IBV strains have undergone divergent evolution paths in Europe and China.

In conclusion, the present study has demonstrated that the circulating IBV strains in commercial flocks in South China had were genetically diverse and underwent continuing evolution; the emergence of new IBV strains will continue to cause persistent but infrequent outbreaks of IBV infections in commercial flocks. Furthermore, the present study challenges the feasibility of inclusion of new variant strains for vaccine preparation for better protection against IBV infection because it is unpredictable of which new variant strains will emerge. Finally, the present study revealed that there were no new predominant IBV strains circulating in the fields in South China yet, but it is reasonable to expect that such predominant IBV strains will eventually emerge under the increased immune pressures due to increased vaccination of commercial flocks. Therefore, this study manifests the importance of continuing surveillance of new IBV strains in order to better prepare for next epidemic or pandemic outbreaks of IBV infections in commercial flocks.

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